The Fibroblast Growth Factor Family in the Early Development of Xenopus laevis.

Thesis

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The Fibroblast Growth Factor Family in the Early Development of *Xenopus laevis.*

Harry Victor Isaacs.
BSc., 1981.
University of Aston.

A thesis submitted to the Life Sciences Department of the Open University for the degree of PhD, May 1996.

Imperial Cancer Research Fund,
Developmental Biology Unit,
Department of Zoology,
University of Oxford.

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Acknowledgements

"Oh! let us never, never doubt

What nobody is sure about!"

Hilaire Belloc, *More Beasts for Worse Children.*

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This thesis is dedicated to Betsy and my mother. I love you both dearly.

Abstract.

The focus of this thesis has been to investigate the requirement for the function of members of the fibroblast growth factor (FGF) family in early *Xenopus* development both during mesoderm formation in the blastula and later patterning events in the gastrula and neurula. In particular, much attention is focused on the activities and expression of *Xenopus* embryonic FGF (*eFGF*) and how this might relate to the perturbations in development which result from the inhibition of the FGF signal transduction pathway following overexpression of a dominant negative FGF receptor (XFD).

A detailed description of the XFD phenotype is presented and shows that inhibition of FGF function leads to a reduction in mesoderm formation and a disruption of its normal pattern. Analysis of gene expression in embryos injected with XFD mRNA demonstrates that FGF activity is required for the correct regulation of a subset of genes within the mesoderm at the start of gastrulation. Prominent amongst these is the *Xenopus* homologue of the *Brachyury* gene (*Xbra*). A detailed *in situ* hybridisation analysis shows that eFGF is coexpressed with *Xbra* in the mesoderm of the periblastopore region and notochord during neurula and gastrula stages. Later in development they are both expressed in the developing tailbud. *FGF-3* is also expressed in the nascent mesoderm and tailbud but also has complex expression domains in the anterior of the embryo.

Experiments in this thesis show that not only is FGF function required for the initial expression of mesodermal genes such as *Xbra* and *XmyoD*, but is also required to maintain their expression after the period of mesoderm induction. Furthermore, experiments suggest that *eFGF* and *Xbra* are components of an autocatalytic regulatory loop that is important for the development of the mesoderm in vertebrates. These data demonstrate a role for FGF activity both during the induction of mesoderm in the blastula and its maintenance and patterning during gastrula and neurula stages.

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Chapter 1

Introduction and background.

Xenopus as a model for animal development

The use of amphibians in experimental embryology has a long history, dating back into the last century. In this period many different species have been used but in recent years the amphibian of choice for studies of early development has been the South African frog *Xenopus laevis*. In common with all model organisms used in the study of development, *Xenopus* has its advantages and disadvantages. On the plus side for *Xenopus*, the adults are easy to maintain in large colonies and the females can be induced to lay large numbers of eggs on demand following hormone injection. The eggs can be artificially fertilised to produce large quantities of synchronously developing embryos, which can easily be cultured in simple salt solutions over a wide range of temperatures from 14^0 C to 23^0 C. This allows considerable manipulation of the speed at which development occurs. At 23^0 C the embryos develop rapidly, such that the end of the neurula stage is reached in about 24 hours. At 14^0 C this same developmental stage will not be reached for several days. The embryos themselves are large enough to allow quite discriminating 'cut and paste' style embryological manipulations.

Perhaps the major disadvantage of *Xenopus* is the inability to manipulate its genome easily. The sophisticated methods for manipulating the genomes of genetically tractable organisms, such as *Drosophila*, *C.elegans* and increasingly the mouse and zebrafish, are not possible in *Xenopus*. The reasons for this are simple to understand. The long generation time (18 to 24 months) and large size of the adults renders the saturation mutagenesis screens that have been undertaken for *Drosophila* and zebrafish impractical. Superimposed upon this is the pseudo-tetraploid nature of the *Xenopus* genome. This means that most

genes are represented by two so-called pseudoallelic variants which differ by up to 10% at the amino acid level. The presence of two allelic and two pseudoallelic copies of a particular gene in each somatic cell clearly complicates any genetic manipulation (Kobel and du Pasquier, 1986), be it through the induction of mutations or through some form of directed reverse genetics.

Despite the lack of the powerful genetic techniques, which have provided the basis for the understanding of *Drosophila* development, *Xenopus* has become an increasingly popular system for the analysis of early development. The last 10 years has seen a huge increase in the understanding of inductive processes in vertebrate development and much of this comes from work done in *Xenopus*. Historically, the amenability of amphibians, such as *Xenopus*, to cut and paste embryology means that they have proved particularly useful in the study of the cell interactions which are so important in the developing vertebrate embryo. The ability to explant groups of cells and allow them to develop in isolation or to juxtapose particular groups of cells has helped define inductive processes involved in the formation of not only the major body axes and germ layers but also individual organs such as the eye and heart (Sater and Jacobson, 1989; Henry and Grainger, 1990; Kessler and Melton, 1994). The major advances in recent years have come from the identification of purified factors that are able to mimic the endogenous inductive processes. Moreover, techniques of molecular cloning and analysis of gene expression have actually identified some of these factors as candidates for fulfilling the role of endogenous inducers (reviewed by Kessler and Melton, 1994; Slack, 1994).

The development of sensitive techniques for the detection of gene expression has for the first time provided workers with markers of regional specification that has allowed the detailed analysis of the very earliest responses to inductive signals during embryogenesis. Molecular biology has also provided *Xenopus* workers with their own form of 'pseudogenetics'. The large size of *Xenopus* embryos makes possible the simple injection of synthetic mRNAs and DNA constructs that allow the ectopic overexpression of particular gene products during early development. The imaginative use of these techniques has allowed the development of innovative screening strategies, which has not only resulted in the identification of novel molecules and activities but has even allowed the inhibition of the function of a wide range of gene products, allowing the type of analysis that was only previously possible in genetically tractable organisms (Amaya *et al.*, 1991; Hemmati-Brivanlou and Melton, 1992; Graff *et al.*, 1994).

The development of more and more sophisticated methods for regulated overexpression and inhibition, used in conjunction with classical embryological techniques will guarantee an increase in the understanding of inductive processes in early *Xenopus* development. Work in the last decade has served to emphasise the large degree of conservation of developmental mechanisms across animal groups, making it very likely that much of what is discovered in *Xenopus* will be directly applicable to the understanding of development in the higher vertebrates.

It is not the intention of this introduction to give a detailed account of the latest understanding of all the inductive events and candidate molecules involved in regional specification in *Xenopus*, where appropriate this is dealt with in detail in the experimental chapters. The introduction will provide a background to this

thesis and include an overview of the properties of the fibroblast growth factors and their receptors.

Background to this thesis

Much work on *Xenopus* in recent years has concerned the development of the mesoderm during blastula stages. Formation of the mesoderm is one of the earliest patterning events in the development of *Xenopus*. It is believed to arise as the result of a set of inductive interactions involving signals emanating from the unpigmented vegetal hemisphere of the embryo acting upon cells competent to respond in the equatorial region of the embryo. The process was originally defined by classical embryological methods but the study of this process has greatly benefited from the advances in technology described above (reviewed by Slack, 1994).

In 1987 members of the fibroblast growth factor (FGF) family of polypeptides were shown to be capable of mimicking some aspects of the endogenous mesoderm inducing signals (Slack *et al.*, 1987). This demonstration not only allowed these factors to be used to investigate the process of mesoderm induction but also established them as candidates for the endogenous inducers. This candidacy was strengthened when members of the family were shown to be present during the early development of *Xenopus*, mouse and chick (reviewed by Slack, 1994; Yamaguchi and Rossant, 1995). Furthermore, it was shown that the inhibition of FGF function during early development by the overexpression of a dominant negative receptor (XFD) led to a reduction in the amount of mesoderm and a derangement of its pattern (Amaya *et al.*, 1991). The initial interpretations

of how this phenotype arises were strongly influenced by the view that an FGF, in particular basic FGF (bFGF), was likely to be one of the vegetal inducers. At the time that work on this thesis commenced this was the prevailing view in the literature (Gilbert, 1988).

However, in 1992 when work began on this thesis new data suggested that this interpretation was overly simple and pointed to the need for further work. Firstly, although bFGF was the first purified factor shown to have mesoderm inducing activity and to be present in the early embryo (Kimelman *et al.*, 1988; Slack and Isaacs, 1989), in common with a number of other FGFs, it lacks a recognised secretory signal sequence. Work carried out in this laboratory indicated that for this reason bFGF is inefficiently secreted from embryonic *Xenopus* cells making it an unlikely candidate for one of the vegetal signals (Thompson and Slack, 1992).

Secondly, in 1992 we reported the presence during early development of two new *Xenopus* FGFs (eFGF and FGF-3 (int-2)) with recognised signal peptides (Isaacs *et al.*, 1992; Tannahill *et al.*, 1992). *eFGF* mRNA is present maternally but its expression is not localised to the vegetal hemisphere during blastula stages. This argued against the view that eFGF might be one of the maternal, vegetal inducers. Interestingly, both *eFGF* and *FGF-3* are expressed zygotically. This suggested that certain aspects of the phenotype produced by inhibition of FGF function in early development might be due to an interference with FGF signalling after the period of mesoderm induction. With these new data in mind it was decided to investigate in more detail the requirement for FGF activity during early development.

Objectives of this thesis

The focus of this thesis has been to investigate the nature of the requirement for FGF function in early development both during mesoderm formation in the blastula and later patterning events in the gastrula and neurula. In particular, much attention is focused on the activities and expression of *eFGF* and how these might relate to the perturbations in development which result from the inhibition of FGF function.

The main objectives of this work have been:

1 To characterise the biological activities of eFGF.

2 To extend the initial study of the expression pattern of *eFGF* (Isaacs *et al.*, 1992) and compare it with that of *FGF-3* and the *Xenopus* homologue of the *Brachyury* transcription factor (*Xbra*). The work on *Xbra* was included because the analysis of mice and zebrafish carrying mutations in the *Brachyury* gene suggests that its function is important for the formation of the mesoderm in vertebrates (reviewed Herrmann and Kispert, 1994).

3 To examine in detail the phenotype of embryos overexpressing a dominant negative FGF receptor (XFD), both in relation to morphology and to molecular markers.

4 To establish whether FGFs have functions after mesoderm induction. The original work of Amaya et al., (1991) using XFD is very important and clearly demonstrated a role for FGF function during development. However, given the likely persistence of FGF inhibition resulting from mRNA injections through blastula, gastrula and neurula stages, it was not clear for what developmental processes FGF function was required.

These major objectives have been successfully achieved and experiments relavant to this are described within the chapters of this thesis. It is hoped that the detailed investigation of the properties of eFGF presented here will provide a useful paradigm for FGF function during the development of *Xenopus* and other organisms.

The fibroblast growth factor (FGF) family of ligands

To date nine members of the FGF family of polypeptides have been identified in mammals. FGFs have been isolated from many different vertebrate species and although FGF ligands have not been cloned from the invertebrates, members of the FGF receptor family have been isolated from *Drosophila* and *C.elegans* (Shishido *et al.*, 1993; DeVore *et al.*, 1994). The genes of all FGFs share a three exon genomic structure, underlining their common ancestry. The FGFs are single chain peptides ranging from about 18 KD, for bFGF and aFGF, to over 30 KD for an amino terminally extended form of FGF-3 (int-2). They share between 30 and 80% amino acid residue identity, most of which lies in a core region of about 100 amino acids. Unlike other growth factor families, such as the TGFβs, there are few invariant sequence motifs in the FGFs. The most highly conserved being the sequence FLP at the carboxy terminus of the 'core region'. Although generally there is not a high degree of sequence conservation between individual members of the family, the cross species conservation for a particular FGF is usually very high. For example amino acid identity for bFGF and FGF-9 between *Xenopus* and mouse is 85% and 94% respectively (Kimelman *et al.*, 1988; Song and Slack *et al.*, 1996)

The prototypes of the family are acidic (aFGF) and basic FGF (bFGF). They were originally purified from a number of sources as mitogens for a wide range of cell types including mouse 3T3 cells, endothelial cells and chondrocytes. In common with all other members of the family they bind to heparin and heparan sulphate residues, which has important consequences for the way that these factors interact with the extracellular matrix but also provides a convenient means of purifying these factors. An important feature of the FGF family is that some FGFs (aFGF, bFGF and FGF-9) lack recognised secretory signal peptides. This issue will be dealt with in detail in subsequent chapters.

Since the identification of aFGF and bFGF, seven further mammalian FGFs have been identified by various means. Keratinocyte growth factor (KGF), androgen induced growth factor (AIGF) and glia activating factor (GAF), like bFGF and aFGF, were identified and purified as proteins on the basis of biological activities (Miyamoto *et al.*, 1993; Ohuchi *et al.*, 1994). *hst/ks* (*kFGF*), *int-2* and *FGF-5* were all identified as protooncogenes. Only *FGF-6* was cloned by homology to a known FGF, in this case *kFGF* (Yoshida *et al.*, 1987; Marics *et al.*, 1989; Dickson, 1990). From the above list it can be seen that the nomenclature for the FGFs is at best confusing. An attempt has been made to rationalise this situation and a table of old and new names is provided below (Baird and Klagsburn, 1991).

On the whole this new nomenclature has been successful and has been adopted by most workers in the field but in the case of *Xenopus* eFGF the assignment of a direct mammalian homologue has not been possible so in this thesis the name embryonic FGF (eFGF) has been retained.

Table 1 The new nomenclature for the FGF family of ligands

Old name	New name
acidic FGF	FGF-1
basic FGF	FGF-2
int-2	FGF-3
ks/hst/kFGF	FGF-4
FGF-5	FGF-5
FGF-6	FGF-6
Keratinocyte growth factor (KGF)	FGF-7
Androgen induced growth factor (AIGF)	FGF-8
Glia activating factor (GAF)	FGF-9

Expression and biological activities of the FGFs in the adult

The whole issue of embryonic expression and biological activities of FGFs during development forms the basis of this thesis and will be dealt extensively in later chapters. In the adult the most abundant FGFs are FGF-1 and FGF-2. They are almost ubiquitous in their distribution. However, they are most highly expressed in the brain and are found in both specific neuron populations and supporting glia cells (reviewed by Basilico and Moscatelli, 1993). FGF-5 is also expressed at low levels in the adult brain but only in specific subsets of neurons (Guo *et al.*, 1996). The FGFs support the survival in culture of neurons from many different sites in the brain and it has been proposed that an important function of the FGFs in the adult is as neurotrophic factors promoting neuron survival and neurite outgrowth. There is also some evidence that FGF-5 in skeletal muscle of the adult can act as a target derived trophic factor for motor neurons (Hughes *et al.*, 1993).

Of course FGF-1 and FGF-2 lack signal peptides, and it has been suggested that the main route of release for these factors is through damage to cells. This is in keeping with the finding that the FGFs have potent angiogenic

activity and have been shown to improve wound healing in the adult . The role of the FGFs in angiogenesis not only extends to stimulation of endothelial cell proliferation but FGF-2 has also been shown to induce an invasive phenotype in endothelial cells allowing them to penetrate basement membranes through an increased production of proteolytic enzymes (reviewed Folkman and Klagsbrun, (1987)). Although cell trauma is likely to be an important pathway for FGF release in the adult, there is some evidence that FGF-1 can be released from cells by a novel mechanism in response to heat shock (Jackson *et al.*, 1992).

Intriguingly, there is accumulating evidence that some of the activities of the FGFs do not require release of the ligand from the cell or interaction with cell surface receptors but are mediated at the level of the nucleus. N-terminally extended isoforms of FGF-1, FGF-2 and FGF-3, which are translated from a CUG codon upstream of the normal translation start site, have been shown to translocate to the nucleus of certain tissue culture cells. In the case of FGF-1 and FGF-2, this not only occurs in cells which are synthesising the protein but exogenously added protein will also translocate to the nucleus (reviewed by Mason, 1994). Nuclear accumulation of FGF-2 has also been shown to occur in the *Xenopus* blastula and epiblast cells of the chick prior to gastrulation (Riese *et al.*, 1995; Song and Slack, 1994). There are also suggestions as to the function of the FGFs at the level of the nucleus. The addition of FGF-2 to isolated nuclei from ABAE cells results in an increase in transcription of ribosomal RNA genes. It has also been shown that targeting of a diphtheria toxin-FGF-1 fusion to the cytoplasm and thence to the nucleus, in cells lacking an FGF receptor, stimulates the synthesis of DNA in the absence of protein phosphorylation that is normally

associated with activation of the cell surface signal transduction pathway (Wiedlocha *et al.*, 1994).

Of the known FGFs the properties of FGF-7 (KGF) are perhaps most divergent. Unlike the other FGFs it is not a mitogen for endothelial cells. It is a potent mitogen for keratinocytes, and in keeping with this, it is highly expressed in the dermis (Mason *et al.*, 1994).

The FGFs as oncogenes

Clearly almost any gene with the mitogenic activity of the FGFs has potential as an oncogene and in fact 3 FGFs were originally identified as oncogenes. *FGF-3* (then *int-2*) was shown to be a gene that was activated by insertion of the mouse mammary tumour virus (MMTV). *FGF-4* (then *hst/ks* or *kFGF*) has also been shown to be activated by the MMTV but was originally identified as a DNA capable of transforming NIH 3T3 cells from human stomach tumours (hst) and Kaposi's sarcoma (ks) cells. *FGF-5* was also identified in a similar transformation assay. In these cases the mode of activation of these genes appears to be at the level of gene regulation rather than the formation of mutant proteins

As has been mentioned *FGF-3* and *FGF-4* clearly have oncogenic potential and furthermore, have both been found to be activated in a number of mouse mammary tumours. In humans, although these genes have been shown to be amplified in a large number of tumours, including breast tumours, mRNA expression is only infrequently detected. It has been suggested that the explanation of this lies in the fact that FGF-3 and FGF-4 are closely clustered on chromosome 11 and that in the tumours they are coincidentally amplified because

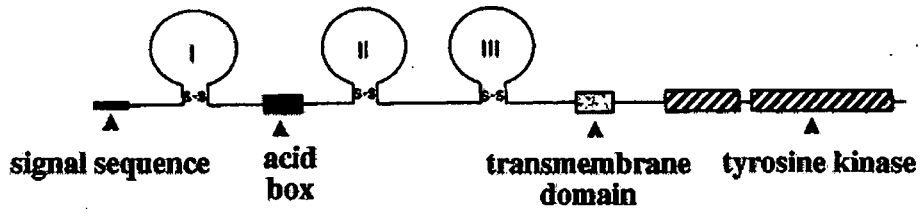
of the nearby presence of another gene which is the actual oncogene involved in the initiation of these tumours. FGF-2 is commonly expressed at high levels in malignant melanomas and is a potent mitogen for melanoma cells. However because of the issue of secretion the relevance of this to the progression of melanomas is at present unclear (reviewed by Basilico and Moscatelli, 1993).

Despite the caveats described above it does seem likely that the mitogenic activity of the FGFs is important for the development of at least some cancers. It is also likely that the potent angiogenic activity of the FGFs plays a role in the development of a blood supply to certain solid tumours. However, the FGFs are just one of many factors which are required in carcinogenesis and the development of the malignant phenotype (reviewed by Basilico and Moscatelli, 1993).

The FGF receptor (FGF-R) family

The high affinity cell surface receptors for the FGFs have been identified as members of the tyrosine kinase receptor family. Four members of the FGF-R family have been identified to date in mammals. The basic structure of an FGF receptor is shown in Fig. 1. The extracellular part of the molecule resides in the amino terminal half and consists of 3 immunoglobulin-like (Ig) domains, with the so-called acid box residing between loops I and II. The acid box which consists of 8 consecutive acid residues is a unique feature of the FGF-R family, the function of which is at present unclear. There is a single membrane spanning domain and a characteristically split intracellular tyrosine kinase domain.

Fig. 1 A schematic diagram of a generalised fibroblast growth factor receptor



Each of the FGF-Rs identified, except FGF-R4, exist in a bewildering number of alternatively spliced isoforms, the relevance of which is only now beginning to be understood. So for example, isoforms of FGF-R1 and FGF-R2 been identified that lack the 'Ig' loop I. This deletion does not abolish ligand binding but does affect the binding affinities and specificity of the ligands for this isoform. A secreted form of FGF-R1 lacking all of the transmembrane and kinase domain has also been identified. However, the most well characterised differences between the various isoforms involves alternative splicing in the second half of 'Ig' loop III and it seems that alternative splicing into this loop to a large extent determines the binding affinity of the various FGF ligands to the receptor isoforms. In FGF-R1 three different exons (a, b and c) have been shown to be spliced into the second half of loop III. In FGF-R2 and FGF-R3 IIIb and IIIc isoforms have been identified. At present only the cDNA for the IIIc isoform of FGF-R4 has been isolated. The table shows the available data for the binding of the various ligands to the receptor Isoforms. This further complicated by the ligands having varying affinities for the different receptors that they have been shown to bind (reviewed by Johnson and Williams, 1993).

Table 2 Receptor binding specificity of the FGF ligand family.

Ligand	FGF-R1	FGF-R2	FGF-R3	FGF-R4
FGF-1	IIIa, b and c	IIIb and c	IIIb and c	IIIc
FGF-2	IIIb and c	IIIc	IIIc	IIIc
FGF-3	No	IIIb and c	No	No
FGF-4	IIIb and IIIc	IIIb and c	?	?
FGF-5	?	?	?	?
FGF-6	?	?	?	?
FGF-7	No	IIIb (-Ig loop I)	?	?
FGF-8	No	IIIc	IIIc	IIIc
FGF-9	No	IIIc	IIIb and c	?

Clearly this list is not complete but it does serve to illustrate the huge complexity of interactions that are possible between the different FGFs and the various FGF receptor isoforms.

I n common with many growth factor receptors, the FGF-Rs bind their ligands as dimers. Dimerisation of the receptor leads to the autophosphorylation of receptor components on a number of tyrosine residues. The activated receptor complex is then able to bind src homology (SH2) domains within a number of intracellular components at these phosphorylated sites. Some of these intracellular components are themselves catalytic and can have their enzymic activity modified by phosphorylation from the tyrosine kinase of the receptor complex (reviewed Egan and Weinberg, 1993). Such enzymic components can include phospholipase c-gamma (PLC-γ) and the GTPase ras. The signal transduction pathway involving ras is of crucial importance in mediating the mesoderm inducing activity of the FGF family and this will be dealt with in some detail in subsequent chapters. Phosphorylation of PLC-γ leads to an increase in

its ability to catalyse the hydrolysis of phosphatidylinositol bisphosphate to inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG), which in turn cause the release of Ca^{++} from intracellular stores and the increased activity of protein kinase C. However, the increase in phosphinositide hydrolysis does not occur in all FGF responsive cells and so the role of PLC-γ activation in FGF signal transduction is at present unclear (Johnson and Williams, 1993; Clyman *et al.*, 1994; Muslin *et al.*, 1994a; Ryan and Gillespie, 1994).

As for down steam target genes of FGF signalling, in systems where the FGFs act as mitogens, the transcription of *c-myc* and *c-fos* is frequently stimulated. The down stream target genes of FGF signalling during mesoderm induction are discussed in detail in subsequent chapters.

Binding of the FGFs to heparan sulphate residues on proteoglycan low affinity receptors in the extracellular matrix greatly increases their affinity for the tyrosine kinase signalling receptor. The high affinity receptors also bind heparin and it has been suggested that a tripartite interaction of this sort may be a prerequisite for the activation of the receptor-ligand complex (for discussion see Mason, 1994).

As with the ligands, the FGF-Rs are expressed in complex and overlapping expression patterns in the adult. Expression of *FGF-R1* is found in bone, kidney, skin, lung, heart, muscle and in the neurons of the CNS. *FGF-R2* is expressed in the skin, lung, liver and in the glia of the CNS. *FGF-R3* mRNA is also expressed in the brain, kidney, skin and lung (reviewed by Johnson and Williams, 1993).

Given the overlap of expression between the various receptors and ligands and the considerable cross reactivity between the ligands and receptors it can be

seen that the FGF signalling system in the adult is enormously complex. During embryonic life the situation is no less complex because all the receptors and ligands described above are also expressed in elaborate and dynamic patterns. In mouse the requirement for the FGF system is being addressed by the generation of null mutations for individual components. The results from these experiments are summarised in Chapter 8. However, it is clear that in the mouse there is a very early requirement for FGF activity to support the growth of the inner cell mass (ICM) which complicates the analysis of the role of the FGF in inductive and patterning events during early development (Feldman *et al.*, 1995). This requirement to support cell proliferation is absent during very early amphibian development. This fact combined with the advantages of the amphibian embryo for micromanipulation and the study of inductive interactions makes *Xenopus* an ideal system in which to study the role of the FGFs in early patterning events. The rest of this thesis is a description of experiments which address this issue.

Published work of the author relevant to this thesis.

My work involved in the cloning and preliminary description of the expression pattern of *Xenopus eFGF* (Isaacs *et al.*, 1992) and the initial description of the expression pattern of *Xenopus FGF-3* (Tannahill *et al.*, 1992) was undertaken prior to registration for this degree. These two papers cannot be considered as part of the work contributing to this thesis and for that reason have not been included, although a brief description of the cloning of *eFGF* has been included in the methods of Chapter 3 for the benefit of the reader.

Some of the work undertaken in the thesis has been published. The data relevant to the role of eFGF in regulating *Xbra* expression during gastrulation was published in Isaacs *et al.*, (1994). All work in this paper is that of the author except the plasmid based embryo injections and their analysis, which was undertaken by M.E.Pownall. Part of the whole-mount *in situ* hybridisation analysis of *eFGF* expression has been published (Isaacs, *et al.* 1995). All work in this paper is that of the author except the *Xbra in situ* data, which was undertaken by M.E.Pownall. The Einsteck-procedure analysis of eFGF treated animal caps is the work of the author and has been published as part of the study in Slack and Isaacs, (1994). Some data from the whole-mount *in situ* analysis of anteroposterior markers in embryos injected with dominant negative FGF receptor mRNA have been submitted as part of (Pownall *et al.*, submitted).

Chapter 2

General materials and methods.

General Embryological methods

Egg production

Egg production is induced by the sub-cutaneous injection of 150-750 iu of human chorionic gonadotrophin (Intervet) into wild-type and albino female *Xenopus laevis*. The amount injected is adjusted according size of the individual and previous egg production record. Empirically, it has been found that albino females require much less hormone to stimulate good egg production. Following injection the females are incubated at 20° C overnight (16-20 hours). The following day eggs are squeezed from the female by applying gentle pressure to the abdomen. The eggs are placed in a petri dish and are then teased into a monolayer and fertilised with a sperm suspension, obtained by crushing a piece of fresh testis in a small volume of distilled water. 5 minutes after the application of the sperm suspension the petri dish is flooded with water. After a further 15 minutes at 23° C a successful fertilisation is apparent by the rotation of the zygotes within the vitelline membrane such that the pigmented animal hemisphere comes to rest uppermost. This procedure allows the production of large quantities of synchronously developing embryos.

The jelly coat of the embryos is removed at an appropriate stage by gently swirling the embryos in 25-50 ml of 2.5% cysteine hydrochloride pH 7.8 for a few minutes. Once it is apparent that the jelly coat has been removed the embryos are washed several times in aquarium water. Following the removal of the jelly coat the embryos are transferred to NAM/10 in noble agar coated petri dishes and cultured at 14° C - 23° C until the required stage of development is reached.

Normal amphibian medium (NAM) based on an analysis of *Xenopus* blastocoelic fluid (Slack *et al.*, 1973) is the standard amphibian saline use in this study.

Normal amphibian medium (NAM) salts

NaCl	110 mM
KCl	2 mM
$Ca(NO_3)_2.4H_2O)$	1 mM
$MgSO_4.7H2O$	1 mM
Na_2EDTA	0.1 mM

Dilutions of the NAM salts are used according to application. Whole embryo culture is generally carried out in NAM/10 and explant culture in NAM/2. NAM solutions are buffered with 0.05 M HEPES buffer pH 7.5 or 0.05 M phosphate buffer pH 7.5 with the addition of 10 mM $NaHCO_3$ (except NAM/10). Overgrowth of micro-organisms in embryo and explant culture is prevented by the addition of the antibiotic gentamycin at 25 µg/ml.

Embryo staging and morphology

Embryos are staged according to the morphological criteria of Nieuwkoop and Faber, (1967). Hausen and Riebesell, (1991) is used as additional reference on embryo morphology and histology. *Xenopus* embryos can be grown at a wide range of temperatures from 14° C to 23° C and manipulation of incubation temperature allows considerable control over the rate at which the embryos develop.

A number of experiments in this study have relied on being able to determine the dorsal side from ventral side of the embryo at the 4-cell stage. At

this stage, as viewed from the animal pole, the two blastomeres on the future dorsal side of the embryo are lighter than those on the ventral side and are generally smaller. The ability to accurately assess presumptive dorsal and ventral sides is very much embryo batch dependent. Only batches of embryos where this assessment can be made accurately are used in experiments.

Embryo manipulations

Animal cap explants and the serial dilution assay

Embryos are cultured in NAM/10 until the stage appropriate to the experiment, which is usually in the range of stage 8 to 10 (about 5-10 hours after fertilisation at 23° C). The embryos are transferred to NAM/2 just prior to operating and the vitelline membranes are carefully removed using sharpened watch makers forceps. A cap of tissue, subtending about 60 degrees, is removed from the animal pole region using an electrolytically sharpened tungsten needle. Care is taken not to contaminate the explant with cells from the bottom of the blastocoel and the marginal zone. The tissue explant is left to recover for 15 to 30 minutes in NAM/2. After this period the explant is gently pipetted up and down to remove dead cells. Animal caps are then transferred to the final culture medium and allowed to develop until an appropriate stage.

The serial dilution cap assay is essentially as described in Godsave *et al.*, (1988). It is generally carried out in the wells of Terasaki microtitre plates (Sterilin). The bottoms of the wells to be used are coated with 3 µl of 15 molecular grade agarose. 10-15 µl of serial dilutions of the test protein solution in NAM/2 + 5% bovine serum albumin (BSA) are placed into the wells of the microtitre dish.

Appropriate negative control solutions are always included in such an assay. The animal cap explants from stage 8 embryos are then placed in the wells by transfer with a blunt ended Gilson pipette tip. Care is taken such that the blastocoel surface of the explant is flipped upwards to ensure maximum exposure to the test factor solution. Animal caps are taken at stage 8 for this assay because they are maximally competent to respond to mesoderm inducing signals at this stage. The ability to respond to mesoderm inducing signals falls off rapidly after stage 10.5 (Jones and Woodland, 1987). The Terasaki dish is placed in a damp chamber and the explants cultured are allowed to develop for an appropriate period. The explants can be visually scored for mesoderm induction after 24-72 hours of culture at 23° C. Alternatively they are harvested for molecular or histological analysis at an appropriate stage of development. If culture is extended beyond 24 hours the wells of the dish are topped up with water to make good any losses from evaporation. Using this assay the mesoderm inducing activity of a given solution is defined as the reciprocal of the last positive dilution per ml. In a similar way the specific activity of a purified protein is defined in units per mg.

The auto induction assay

The autoinduction assay is a development of the serial dilution assay which is used to test the activity of injected synthetic mRNAs, where it is assumed that the activity is proportional to the mass of mRNA injected. The animal cap explant procedure is slightly modified for animal caps that are removed from embryos which have been previously microinjected with RNA samples. This is to ensure that the blastocoel does not collapse following injection and exposure to Ficoll

containing solution. The embryos are injected in NAM + 5% Ficoll solution (see below) and allowed to develop until the 64 to 128 cell stage at which time they are transferred to NAM + 2.5 % Ficoll. Animal caps are taken from embryos between stage 8 and 10 and then cultured in microtitre plates and analysed as in the serial dilution assay.

Disaggregated cell culture of gastrula stage explants

At gastrula stage 10.5 embryos are dissected into dorsal and ventral halves. Animal hemisphere tissue is then removed down to the floor of the blastocoel and as much as possible of the vegetal core material is dissected away. The marginal zone pieces to be disaggregated are then allowed to sit for 20 minutes in agarose coated dishes containing Ca^{++}/Mg^{++} free NAM (CMF-NAM) and are finally transferred to agarose coated wells of 4-well tissue culture plates (Sterilin) for disaggregation and culture. Explants are disaggregated by gentle pipetting using drawn glass Pasteur pipettes. All agarose dishes are extensively pre-washed and equilibrated with culture medium, which is generally CMF-NAM + 5% BSA + relevant factors.

The Einsteck-procedure

The Einsteck-procedure is any embryo manipulation that involves the placing of a tissue explant or pelleted substance into the blastocoel of a developing embryo. Generally all tissue explants used in the Einsteck-procedure are taken from embryos which have been previously injected with a fluorescein-dextran-amine lineage label (Molecular Probes). The lineage labelling of the donor tissue greatly

enhances the value of this technique and enables the rapid recognition of graft and host derived structures during subsequent analysis by fluorescent microscopy on whole-mount or sectioned material.

Briefly, donor tissue explants are taken from the required stage of labelled embryo in NAM/2 using either tungsten needles or a microsurgical knife (Microsurgical Products, Inc.). Host embryos are cultured in NAM/10 until the dorsal lip is first apparent at stage 10 and are then transferred to NAM/4 prior to operating. Hosts are carefully demembranated and a small slit is placed in the animal pole region at right angles to the dorsal ventral axis and the donor material is placed into the centre of the host blastocoel. Following implantation the Einsteck-hosts are placed carefully aside, making sure that they remain animal pole up. The blastocoel roof is allowed to heal, which normally takes about 30 minutes. At this stage the embryos are transferred to NAM/10 for further incubation at the required temperature. This procedure ensures a good rate of success and minimises the number of embryos that fail to gastrulate following the Einsteck-procedure.

Microinjection of embryos

All injections are carried out using a Drummond "Nanojet Variable" automatic injector mounted on a Prior micromanipulator. This system provides variable injection volumes from 5 nl to 75 nl in 5 nl steps. The system uses oil filled needles and a positive displacement plunger system that provides reproducible results and does not require frequent recalibration. Needles are pulled from Drummond 3.5" inch capillaries using a Scientific Research Instruments vertical

needle puller. Before use needles are baked overnight at 180^0 C to destroy RNAases. Sterile light mineral oil (Sigma) is used to back-fill the needles and the tips broken off to give an external diameter of approximately 20 µm. Samples are loaded into the needle by sucking up from a droplet of sample placed the inside surface of a sterile petri dish. Following the loading of the sample to be injected the interface between oil and aqueous phases in the needle provides an excellent method of monitoring the volume of liquid expelled from the needle during injection.

Before injection embryos are dejellied and cultured in NAM/10 until the required stage. A few minutes before injection begins the embryos are transferred to an agar coated dish containing NAM + 5% Ficoll 400 (Sigma). Ficoll is a high molecular weight non-toxic carbohydrate which osmotically removes water from the perivitelline space. The presence of Ficoll collapses the vitelline membrane down on to the embryo surface and in this way the embryo is no longer able to rotate freely within its vitelline membrane. This greatly increase the ease of accurate injection and also reduces the amount of cytoplasmic blebbing from the injection wound site. The embryos are placed in a trough in the agar dish to provide support and are injected using gentle pressure from the micromanipulator. The needle is retained in the embryo for several seconds following expulsion of liquid to allow dispersion from the site of injection.

Following injection the embryos continue to be cultured in NAM+5% Ficoll until the 64-128 cell stage. If the embryos are required for later removal of animal cap explants at stage 8 to 11 they are then tranferred to NAM + 2.5% at this stage. This is to prevent the collapse of the blastocoel, which can occur at the

higher Ficoll concentration. For all other purposes the embryos are transferred to NAM/10 + 5% Ficoll. The continued presence of Ficoll reduces the amount of cytoplasmic blebing from the injection wound and does not adversely effect normal development.

Injection of fluorescent lineage labels

Fluorescein dextran amine (FDA) (10,000 mw) from Molecular Probes is prepared for injection by dissolving at 25 mg/ml and then dialysing overnight against water. Aliquots are stored at -80° C until required. Complete and reproducible labelling of the entire embryo is obtained by injecting 15 nl of stock FDA into the animal pole region just after the onset of first cleavage. Following injection all embryos are examined under the fluorescent microscope to ensure that they are labelled.

Injection of synthetic mRNAs

Particular attention is paid to maintaining RNAase free conditions when handling RNA samples for microinjection. Stock aliquots of synthetic mRNAs in diethylpyrocarbonate (DEPC) treated water are prepared at 0.2 µg/µl and stored at -80° C until required. Just before injection the mRNAs are diluted with DEPC water to their final concentration. At the 2-cell stage a maximum of 10 nl/blastomere of sample is injected and a maximum of 5 nl/blastomere at the 4-cell stage.

General histological methods

Preservation of specimens for whole-mount examination

Specimens are fixed 4% formaldehyde in 70% Dulbeccos's phosphate buffered saline 'A' (PBS-A). Specimens are stored in the above fixative in the dark to avoid photobleaching of pigment.

Preparation of stained histological sections

Specimens are fixed for at least 4 hours in 4% formaldehyde in 70% PBS-A. They are then washed in 70% PBS-A and equilibrated with 35% industrial methylated spirits (IMS) before staining overnight with a 10% solution of borax carmine in 35% IMS. The next day specimens are destained for at least 4 hours with 1% HCl in 70% IMS. At this stage specimens are transferred to 70% IMS and can be stored like this until required for further processing.

Specimens are taken through a water/IMS/n-butanol series and embedded in paraffin wax (Pastillated Fibrowax, BDH). Wax blocks are mounted on wooden chucks and 10 µm microtome ribboned sections are taken. Sections are floated on warm water to remove wrinkles and dried down onto gelatin subbed glass slides. Once dry wax sectioned are briefly melted over a Bunsen burner flame and slides are dewaxed and rehydrated through a Histoclear (National Diagnostics)/ IMS/water series. Once sections have been taken to water they are then counterstained for 1 minute with a 0.025% aqueous solution of napthalene black saturated with picric acid. Excess stain is washed off in water and then

slides are dehydrated through a water/IMS/Histoclear series and mounted in DePeX (BDH).

Preparation of fluorescently labelled sections

FDA lineage labelled specimens are embedded and sectioned as described above. Sections are taken to water and stained with a 1 µg/ml aqueous solution of the DNA binding dye DAPI for 10 minutes. Excess DAPI solution is washed off with water and slides are taken to Histoclear and mounted in DePeX. The use of DAPI causes the nuclei within sections to fluoresce a bright blue when excited in the range 365- 376 nm.

General photographic methods

Black and white photographs of whole-mount specimens are taken using Kodak Technical Pan film rated at 260 ASA or Kodak TMAX-400 film. Black and white photographs of histological specimens are taken using Kodak TMAX-100 and TMAX-400 film. Autoradiographs and autofluorographs are illuminated by transmitted light and photographed with Kodak Technical Pan film. All films are developed as per manufacturers recommendations.

Colour slide photography of specimens are carried out using Kodak Professional Ektachrome 160T film and was developed using E6 processing. All prints are prepared by electronically scanning colour slides with a Nikon Coolscan scanner. Images are processed using Adobe Photoshop and printed on a Kodak XLS8600 photo-quality printer.

General molecular biological methods

Standard protocols for nucleic acid quantification, handling and purification such as phenol/chloroform extraction and ethanol precipitation are as per Sambrook *et al.*, (1989). All RNA related work requires standard precautions related to the maintenance of RNAase free conditions. These include the use of gloves, diethylpyrocarbonate (DEPC) treated water, where possible, and the use of baked glassware and sterile RNAase free plastics.

Production, analysis and manipulation of DNA

Restriction enzyme analysis

Carried as per standard methods (Sambrook *et al.*, 1989) and manufacturers recommendations (Boehringer, New England Biolabs, Promega and Stratagene).

Gel electrophoresis of DNA

DNA gels are analysed in ethidium bromide stained 0.8% to 2% agarose (Life Technologies) horizontal submarine gels using standard protocols (Sambrook *et al.*, 1989).

Isolation of DNA fragments from agarose gels

Slices containing the required DNA fragment are cut from agarose gels under long wave UV illumination to avoid short wave UV damage to the DNA. Purification of the DNA is carried out using the glass filter based Glass-Max DNA (Life Technologies) isolation kit.

Ligation of DNA into plasmid based vectors

Digested DNA fragments and vectors are prepared and purified as described above. Vectors are generally desphosphorylated with calf-intestinal alkaline phosphatase (Promega) (Sambrook *et al.*, 1989) to cut down the background of religated vector sequence. Ligations reactions using recombinant T4 ligase (Promega) are set up using standard protocols (Sambrook *et al.*, 1989). Following incubation at 16^0 C for at least 12 hours. 5 µl aliquots of the ligation reactions are used to heat shock transform *E.coli* (strain JM109 or Xl1- blue) (Sambrook *et al.*, 1989). Aliquots of the bacterial transformations are plated on L-agar plates containing 100 µg/ml ampicillin and incubated at 37^0 C overnight. The next day antibiotic resistant colonies are screened by plasmid mini-prep for the presence of recombinant plasmids.

Preparation of plasmid DNA

Large scale preparation of DNA (100 µg to 1 mg) is carried out using the standard alkali lysis method with a final CsCl/ethidium bromide gradient purification (Sambrook *et al.*, 1989). Small scale preparation of DNA (1 µg to 20 µg) is carried out using the glass resin based Wizard mini-prep kit (Promega) as per manufacturers instructions.

Production of synthetic mRNA for microinjection into embryos

All synthetic messages are produced by *in vitro* transcription from cDNAs cloned into the vector pSP64t (Krieg and Melton, 1984). This vector provides a cloned cDNA with 5' and 3' untranslated regions of the Xenopus ß-globin cDNA and a

poly AC tail. The effect of these sequences is to increase the *in vivo* stability and translatability of mRNA synthesised from cDNAs cloned into this vector. All messages for injection are produced using the Ambion SP6 Megascript kit and a modified protocol using 0.5 mM GTP and 5 mM $m^7G(5')Gppp(5')G$. A 1:10 ratio of GTP to cap analogue is used to ensure a high percentage of capped transcript (Krieg and Melton, 1987). Following DNAase treatment to remove template DNA the transcriptions are phenol/chloroform and chloroform extracted. Unincorporated nucleotides are effectively removed by adding ammonium acetate to 0.5 M and precipitating with an equal volume of isopropanol. The integrity of the mRNA is checked by running 1/10 of the transcription product on an ethidium bromide stained 2% agarose gel. Aliquots of the mRNA at 0.2 µg/µl in DEPC treated water are stored at -80° C until required.

Plasmid constructs used in this study to produce synthetic mRNAs

The dominant negative FGF receptor (XFD) and control receptor (d50) constructs are those used by Amaya *et al.*, (1991). The pSP64-ß-globin plasmid is that of Krieg and Melton, (1984). The pSP64-XbFGF plasmid is as described by Thompson and Slack, (1992). The pSP64-eFGF plasmid is as described by Isaacs *et al.*, (1994) and contains the naturally occurring sequence around the ATG initiating codon. The pSP64-eFGF (mod) is as described by Isaacs *et al.*, (1994) and has the sequence around the initiating codon optimised for translation according to the rules of Kozak, (1986). The pSP64-Xbra plasmid is that of Cunliffe and Smith, (1992).

Analysis of mRNA expression in the embryo

Purification of total RNA from embryo material

RNA is extracted by homogenisation of tissues in 0.1 M NaCl, 50 mM Tris (pH 8), 5 mM EDTA and 0.5% SDS followed by phenol/chloroform extraction and ethanol precipitation. This is a simple and efficient method for the recovery of total RNA from embryos and small pieces of tissue such as animal cap explants, suitable for analysis by RNAase protection.

RNAase probe protection analysis

32P-UTP labelled antisense RNA probes are synthesised using ^{32}P UTP 410 Ci/mmol (Amersham). The DNA template is removed by DNAsing and, following the addition of denaturing sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cylanol), the whole reaction is electrophoresed on a 6% polyacrylamide denaturing urea gel. The major radioactive transcript for a given reaction is identified by exposing the gel briefly to X-ray film and the gel region containing the band is cut out and the probe is eluted into 300 µl of 0.5M ammonium acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1% SDS. The probe is ethanol precipitated and redissolved at a final concentration of 25,000 to 100,000 cpm/µl. RNA samples from test tissues are vacuum dried into the hybridisation tubes and the 30 µl hybridisations are set up containing 80% formamide, 0.4 M NaCl, 0.04 M PIPES pH 6.4, 1 mM EDTA and 25,000 cpm of each test probe. If there is sufficient size differences between the final probe protected fragments up to 4 species of mRNA can be reliably analysed simultaneously using this protocol.

Hybridisations are carried out for 12 to 16 hours at 45° C. After hybridisation is complete single stranded RNA and mis-matched RNA hybrids are digested by adding 350 µl of digestion buffer (300 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA containing 700 u/ml of RNAase T1 (Ambion)) and incubating at 37° C for 30 to 60 minutes. RNAse T1 is digested away by adding sodium dodecyl sulphate (SDS) to 0.5% and proteinase K to 125 µg/ml. The digestion mixture is incubated at 37° C for a further 20 minutes and is phenol/chloroform extracted and ethanol precipitated. The ethanol precipitate is resuspended in a small volume of DEPC water and denaturing sample buffer and electrophoresed on a 6% denaturing polyacrylamide. Following electrophoresis the gel is fixed in 10% methanol, 10% acetic acid solution and dried down onto Whatman 3MM paper. Autoradiography is by exposure at -80° C to double screened preflashed XAR X-Omat film (Kodak).

RNAase protection probes in this study

In all RNAase protections the ubiquitously expressed *ornithine decarboxylase* (*ODC*) gene is used as an internal loading control (Isaacs *et al.*, 1992).

Early general-mesodermal markers:-*Xbra* is detected as in Smith *et al.*, (1991). *eFGF* as in Isaacs *et al.*, (1992). *Xsna* (Essex *et al.*, 1993) is detected as in Sargent and Bennett, (1990).

Dorso-ventral mesodermal markers:- *Goosecoid* (Cho *et al.*, 1991) is detected as in Green *et al.*, (1992). *Noggin* expression is detected using Noggin A. This consists of a 740 bp PCR fragment, containing the whole of the coding region of *noggin* (Smith and Harland, 1992) cloned into the EcoRV site of Bluescript II KS+. When linearised with StyI and transcribed with T7 polymerase it

yields a probe giving a full length protected fragment of 243 bases. MyoDb (Harvey, 1991) is used such that only the zygotic expression of *XmyoD* is detected. *Xwnt-8* is detected as in Christian and Moon, (1993).

Antero-posterior markers:- *Xlhbox1* (*HoxC6*)(Carrasco and Malacinski, 1987) is detected as in Cho *et al.*, (1988) using pRII as a probe. *Xhox3* (Ruiz i Altaba and Melton, 1989a) is detected as in Saha and Grainger, (1992). *Xhox36* (*HoxA7*) (Condie and Harland, 1987) expression is detected using the probe Xhox36.4. This consists of an EcoRI/Pst I fragment of the *Xhox36* cDNA cloned into pGEM2. When linearised with EcoRI and transcribed with T7 it yields a probe giving a protected fragment of 231 bases.

In situ hybridisation

Digoxigenin-11 UTP (DIG) (Boehringer) labelled RNA probes are generated as per manufacturer's protocol. DNA template is removed by digestion and the probes are precipitated by the addition of LiCl to 2.5 M. Probes are resuspended in a small volume of 1 mM EDTA and their integrity is checked on a 2% agarose gel. An estimate of probe concentration is made by reference to a DIG RNA standard. Aliquots of probes are stored at -80° C until required. Probes are not hydrolysed before hybridisation.

The *in situ* hybridisation protocol used is based on that of Harland, (1991). The most important change being that embryos are not RNAase treated following hybridisation. This considerably increases the signal strength and generally without an increase in background. Albino embryos are demembranated and in embryos up to tailbud stages the body cavities are punctured to reduce

background due to reagent trapping. Embryos are fixed for 1 hour in MEMFA (0.1 M MOPS pH 7.4, EGTA 2 mM, $MgSO_4$, 1 mM and formaldehyde 4%) with agitation. Embryos are washed for 5 minutes in 100% ethanol and then stored in ethanol at -20° C until required.

Embryos are equilibrated with Dulbecco's phosphate buffered saline 'A' + 0.1% tween (PBS-AT) and are permeabilised by treatment with proteinase K (Boehringer) at 10 µg/ml for 10 to 20 minutes. Wash twice with 0.1 M triethanolamine pH 7.8 for 5 minutes. Acetic anhydride is added to 0.25% to the last wash and the embryos are gently swirled for 5 minutes. Wash twice with PBSA-T for 5 minutes and refix embryos for 20 minutes in PBS-AT + 4% formaldehyde. Wash with several changes of PBS-AT for at east 30 minutes. Embryos are transferred to 1.5 ml screw cap tubes (Sterilin) and equilibrated with hybridisation buffer.

Hybridisation buffer

50% molecular grade formamide (IBI, Kodak-Eastman)
5X SSC (final=0.75 M NaCl, 0.075 Na_3citrate.$2H_2O$ pH 7.0)
1 mg/ml total yeast RNA (ICN)
100 µg/ml heparin (Sigma)
1X Denhardts solution (final/ml=0.1 g ficoll 400, 0.1 g PVP, 0.1 g BSA)
0.1% tween (BDH)
0.1% CHAPS (Sigma)
10 mM EDTA
in DEPC treated water.

Hybridisation tubes are brought to 60° C and embryos are prehybridised with gentle agitation for 2 hours. Hybridisation solution is changed and heat

denatured probes are added to a final concentration of about 1 µg/ml. Hybridisations are incubated with gentle agitation for 16 hours at 60° C.

Probe solution is removed and embryos are washed twice with hybridisation solution at 60° C. Embryos are washed 4X 30 minutes with 2X SSC + 0.1% tween at 60° C. Embryos are washed 4X 30 minutes with 0.2X SSC + 0.1% tween at 60° C. Embryos are equilibrated with MAB-T (100 mM maleic acid, 150 mM NaCl pH 7.8 and 0.1% tween). The solution is replaced with MAB-T + 2% Boehringer DIG blocking reagent and rolled for 30 minutes at room temperature. The solution is replaced with MAB-T + 2% blocking agent + 20% heat treated sheep serum (Sigma) and blocking is continued for a further 90 minutes. Fresh blocking solution is added + 0.05% alkaline phosphatase linked affinity purified sheep anti-DIG antibody (Boehringer) and incubated with gentle agitation for at least 12 hours at 4° C.

Antibody solution is removed and embryos are transferred to 20 ml vials and washed 4X 1 hour with MAB-T. Embryos are equilibrated with fresh alkaline phosphatase buffer (100 mM tris, 50 mM $MgCl_2$, 100 mM NaCl pH 9.5 and 0.1%) tween. Colour reaction is developed in the dark at room temperature for up to 18 hours in a premixed NBT/X-phosphate solution (BM purple AP substrate, Boehringer). After colour development is complete embryos are washed briefly in PBS-AT and then stored in MEMFA. A pinkish background colour can develop after long development times which can be removed by a brief methanol wash.

Embryos are cut open using a microsurgical blade to reveal staining in deep tissue layers. Clearing of embryos to reveal deep tissue staining can be achieved using the following protocol. Equilibrate for 5 minutes in methanol,

followed by 5 minutes in iso-propanol and embryos are finally cleared tetrahydronaphthalene (Aldrich). For long term storage specimens must be returned though an isopropanol/methanol series to MEMFA or PBS-AT to avoid loss of signal.

In situ hybridisation probes used in this study

Xenopus fibroblast growth factors:- *eFGF (i)* is detected using XeFGF (i) GS (Isaacs *et al.*, 1992; Isaacs *et al.*, 1995). It corresponds to 300 bp fragment from the 5' end of the coding region of *XeFGF (i)*. *FGF-3* (*int-2*) as in Tannahill *et al.*, (1992).

Early general-mesodermal markers:-*Xbra* (Smith *et al.*, 1991) is linearised with ClaI and transcribed with T7 polymerase. *Xsna* (Essex *et al.*, 1993) as per RNAase protection probe in Sargent and Bennett, (1990).

Dorso-ventral mesodermal markers:- *Noggin* expression is detected using Noggin A. This consists of a 740 bp PCR fragment, containing the whole of the coding region of *noggin* (Smith and Harland, 1992) cloned into the EcoRV site of Bluescript II KS+. When linearised with StyI and transcribed with T7 polymerase it yields a probe of 243 bases. *Xnot2* as in Gont and De Robertis, (1993).

Antero-posterior markers:- *otx2* is detected as in Pannese *et al.*, (1995). *En-2* and *krox20* as in Doniach *et al.*, (1992). *HoxB1* is detected as in Godsave *et al.*, (1994). *Xhox36* (*HoxA7*) (Condie and Harland, 1987) expression is detected using the probe Xhox36.1. This consists of an Eco RI fragment of the

Xhox36 cDNA cloned into pGEM2. When linearised with Bam HI and transcribed with T7 it yields a probe of 1800 bases.

Tissue type specific markers:- Cardiac *actin* is detected as in Gurdon *et al.*, (1985). *XmyoD* is detected using the RNAase protection probe MyoDb (Harvey, 1991). *Xmyf5* is detected as in Hopwood *et al.*, (1991). *NCAM* (Krieg *et al.*, 1989) is detected as per RNAase protection probe in SchulteMerker *et al.*, (1994b).

Chapter 3

The developmental expression of *eFGF*, *FGF-3* and *Xbra*.

Introduction

The main theme of this thesis is the role that the secreted factor eFGF has during early development. To understand better the nature of this role a detailed study of the expression pattern of this factor was undertaken using whole-mount DIG *in situ* hybridisation. The sensitivity and spatial resolution of the DIG *in situ* protocol has provided a much more detailed view of the expression pattern of *eFGF* than radioactive *in situ* hybridisation on sectioned embryo material and has revealed aspects of *eFGF* expression that were not apparent in the original study (Isaacs *et al.*, 1992).

This chapter also contains a detailed study of the expression patterns of *Xenopus FGF-3* (*int-2*) and *Xenopus Brachyury* (*Xbra*). The expression patterns of these 2 genes have been previously reported (Smith *et al.*, 1991; Tannahill *et al.*, 1992). However, the *FGF-3 in situ* study was included as a comparative exercise because *FGF-3* is the only other known *Xenopus* FGF with a recognised secretory signal sequence. A side by side comparison of the expression patterns of *eFGF* and *FGF-3* is helpful in determining the roles that these factors might have in common. The *Xbra* study was also undertaken for the purpose of comparison because experiments within this thesis and elsewhere strongly suggest a close link between the activities and regulation of the FGFs and *Xbra* (Amaya *et al.*, 1993; Isaacs *et al.*, 1994; Cornell *et al.*, 1995; Schulte-Merker and Smith, 1995).

Xenopus eFGF shares about 60% predicted amino acid identity with mammalian FGF-4 and FGF-6 . The cloning of *eFGF* revealed two closely related cDNAs which share about 95% predicted amino acid identity (Isaacs *et al.*, 1992).

It is likely that these 2 closely related cDNAs (*eFGF(i)* and *eFGF(ii)*) are derived from pseudoallelic genes that arose as the result of a genome duplication event which is thought to have occurred in *Xenopus* about 30 million years ago (Bisbee *et al.*, 1977; Kobel and du Pasquier, 1986). The carboxy terminal halves of eFGF (i) and eFGF (ii) are almost identical and most divergence has occurred in the amino terminus, which includes the secretory signal sequence. However, a potential N-linked glycosylation site close to the predicted signal cleavage site has been retained in both eFGF isoforms. This site is also conserved in mammalian FGF-4 and FGF-6 and indicates that glycosylation has some functional significance for this closely related subset of FGFs (Yoshida *et al.*, 1987; Coulier *et al.*, 1991). However, glycosylation is not a essential for biological activity of these proteins because eFGF and FGF-4 proteins, that have the glycosylation site removed by truncation, retain full biological activity in a number of assays (Isaacs *et al.*, 1992; Bellosta *et al.*, 1993). The nature of the requirement for glycosylation is at present unclear.

The proto-oncogene *FGF-3* was first identified in mouse as a site of insertion for the mouse mammary tumour virus (MMTV) (Dickson *et al.*, 1984; Moore *et al.*, 1986) and homologues have since been identified in a number of vertebrates including human, chick and *Xenopus* (Brookes *et al.*, 1989; Tannahill *et al.*, 1992; Mahmood *et al.*, 1995b). The predicted amino acid sequence of *Xenopus* FGF-3 is approximately 70% identical to human, mouse and chicken FGF-3 sequences. As is the case with eFGF, FGF-3 is glycosylated and has been shown to be secreted from at least some cell lines. (Kiefer *et al.*, 1991). In fact, the *Xenopus* FGF-3 appears to be somewhat more efficiently secreted from

cell lines than does mammalian FGF-3 (Kiefer *et al.*, 1993a). However, unlike *eFGF* only one *FGF-3* pseudoallele has been identified.

The mouse deletion mutant *Brachyury* (*T*) was first described several decades ago. However, it was not until recently that the *Brachyury* gene was identified by positional cloning from the mouse (Herrmann *et al.*, 1990). Since then direct homologues have been identified in *Xenopus*, chicken and zebrafish (Smith *et al.*, 1991; SchulteMerker *et al.*, 1994c; Kispert *et al.*, 1995b). Closely related genes have also been identified in the primitive chordates *Amphioxus* and the ascidian, *Halocynthia roretzi* (Yasuo and Satoh, 1994; Holland *et al.*, 1995).

Brachyury is a member of a family of genes which have been identified in animal species as divergent as the sea urchin, *Drosophila* and man (Kispert *et al.*, 1994; Bulfone *et al.*, 1995; Harada and Yasuo, 1995). They are all putative transcription factors which are characterised by the presence of a conserved 'T-box' DNA binding domain (Kispert *et al.*, 1995a). The expression pattern of *Brachyury* is very similar in all vertebrates examined to date (reviewed by Herrmann and Kispert, 1994). This suggests a highly conserved role for *Brachyury* in vertebrate in development. This view is supported by the analysis of the naturally occurring *Brachyury* mutants in mouse (*T*) and zebrafish (*no tail*), which points to this gene having an important role in the formation of the mesoderm and axial structures (Beddington *et al.*, 1992; Herrmann and Kispert, 1994; SchulteMerker *et al.*, 1994c). Functional studies in *Xenopus* also support this view, and furthermore, strongly suggest a close link between the regulation of *Brachyury* and the activity of the FGFs (Cunliffe and Smith, 1992; Isaacs *et al.*, 1994; Cornell *et al.*, 1995; Schulte-Merker and Smith, 1995).

Materials and methods

Embryological methods

As per general methods.

The cloning of Xenopus embryonic fibroblast growth factor (eFGF)

Xenopus eFGF was cloned from *Xenopus* embryo cDNA using the polymerase chain reaction (Isaacs *et al.*, 1992). Several pairs of degenerate deoxy-oligonucleotide primers were designed from the two mammalian kFGF sequences available (Yoshida *et al.*, 1987; Brooks *et al.*, 1989) and a product of the predicted size was obtained with this primer pair.

XK(10D): 5' TA(T/C)TG(T/C)AA(T/C)GT(I/C)GG(I/C)AT(I/C)GGI 3'

XK(14D): 5' (T/C)TC(A/G)TA(I/C)GC(A/G)TT(A/G)TA(A/G)TT(A/G)TT 3'

Sequence analysis revealed a predicted 70% identity with murine kFGF at the amino acid level. Using the sequence data obtained from the primary PCR clone primers were designed which enabled the 5' and 3' ends of the cDNA to be obtained by a modified RACE (**R**apid **A**mplification of **C**DNA **E**nds) protocol (Frohman, 1990). Sequence analysis of 5' and 3' RACE products suggested the presence of two closely related cDNA clones. The coding regions of both cDNAs were amplified using primers based upon sequence data obtained from the 5' and 3' RACE products. Several clones of each cDNA, now designated *Xenopus eFGF(i)* and *Xenopus eFGF(ii)* were sequenced on both strands. The nucleotide sequences of both cDNAs are in the EMBL data base. Accession numbers are X62593 and X62594.

In situ hybridisation

Transcriptions and *in situ* hybridisations were carried out as per general methods.

eFGF

The radioactive *in situ* hybridisation study of the spatial expression of *eFGF* used a 250 base probe based on the original product of the degenerate PCR cloning strategy (Isaacs *et al.*, 1992). A number of probes were tested for the DIG whole-mount *in situ* study in this thesis (data not shown). The best signal to noise ratio was obtained using a probe made from eFGF (i) GS (Isaacs *et al.*, 1992). This corresponds to a 300 bp EcoRI/ClaI fragment cloned into Bluescript II KS+ (Stratagene) which covers the 5' half of the coding region from the eFGF (i) cDNA. An antisense probe was made by linearising eFGF (i) GS with EcoRI and transcribing with T3 RNA polymerase. A sense control probe was made by linearising with ClaI and transcribing with T7 RNA polymerase. Colour development was allowed to proceed at room temperature for up to 18 hours.

FGF-3 (int-2)

The *Xenopus FGF-3* probe used in this study is the same as that in Tannahill *et al.*, (1992). It corresponds to a 297 bp EcoRI fragment of the *FGF-3* cDNA coding region cloned into Bluescript II KS+ (Stratagene) which covers all of exon 2 and parts of exon 1 and 3. Antisense probe was made by linearising with BamHI and transcribing with T3 RNA polymerase. Colour development was allowed to proceed at room temperature for up to 18 hours.

Xbra

The *Xbra* plasmid is that of Smith *et al.*, (1991). An 1800 base antisense probe was made by linearising with ClaI and transcribing with T7. Colour development was allowed to proceed at room temperature for up to 4 hours.

Results

Developmental expression of eFGF

eFGF expression in the early gastrula

As discussed above *Xenopus* is pseudotetraploid and as a result 2 copies of *eFGF* are present in the *Xenopus* genome. Interestingly the temporal expression pattern of both *eFGF* pseudoalleles show significant differences. *eFGF (i)* is expressed at considerably higher levels than is *eFGF(ii)* and eFGF(i) is expressed both maternally and zygotically, whereas eFGF(ii) mRNA is only expressed zygotically. However, both *eFGF* isoforms are most highly expressed during gastrula and neurula stages (stage 10 to 19) (Isaacs *et al.*, 1992). It is possible that the 2 pseudo alleles not only have different temporal expressions but also different spatial expression patterns as has been reported for other psedoallelic genes in *Xenopus* (Shuldiner *et al.*, 1991). However, the much lower levels of *eFGF (ii)* expression means that it has proved impossible to obtain any *in situ* data using probes specific to this isoform. All the *in situ* data presented here is obtained using a probe which in RNAase protections is specific to *eFGF (i)*.

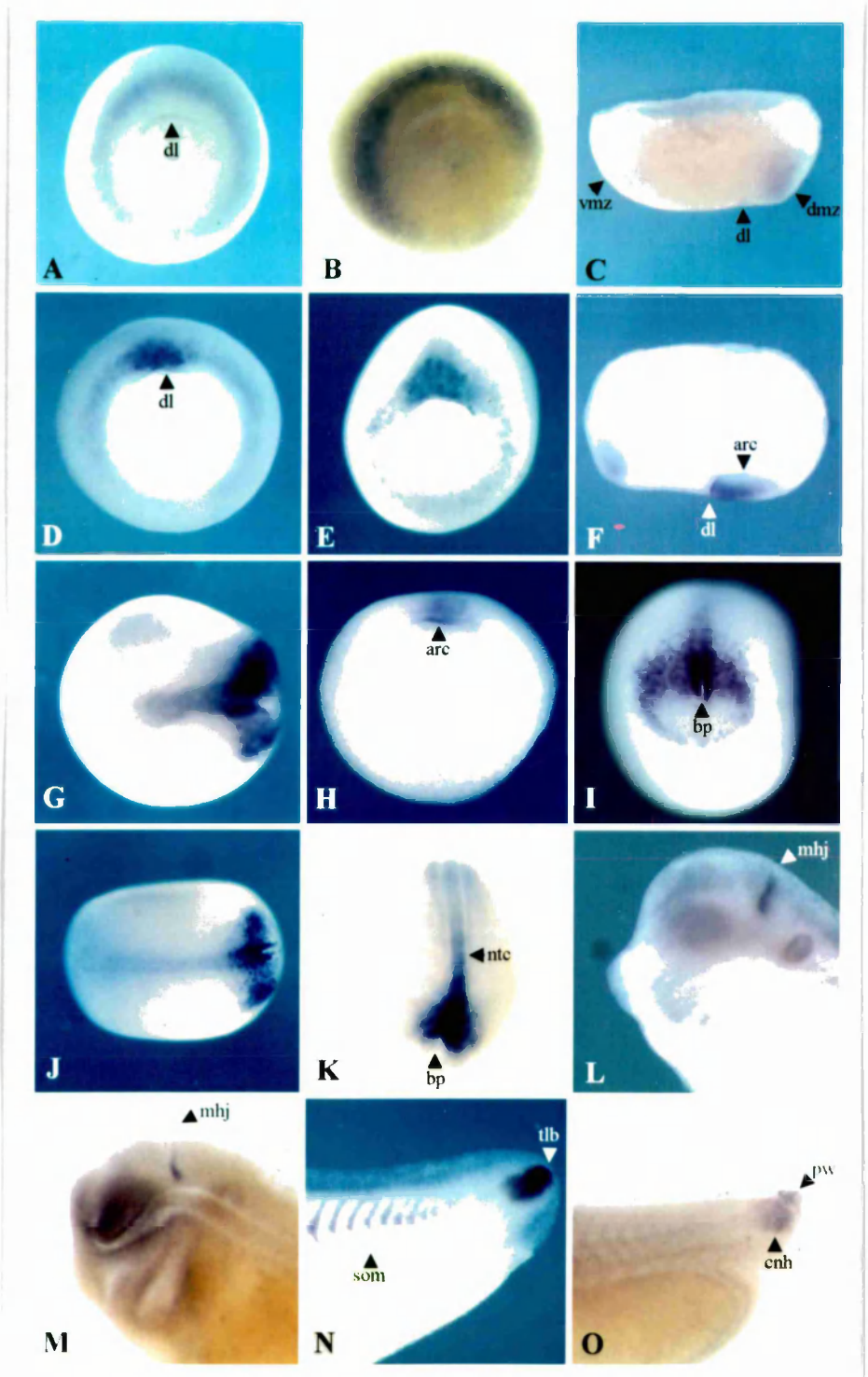
RNAase protection analysis of dissected pieces from blastulae suggest that on a per unit of total RNA basis the maternal *eFGF* mRNA is rather evenly distributed along the animal vegetal axis (Isaacs *et al.*, 1992).

Fig. 1. Whole-mount *in situ* hybridisation analysis of *Xenopus eFGF* expression during normal development.

Abbreviations: **arc**=archenteron. **bp**=blastopore. **cnh**=chordoneural hinge. **dl**=dorsal lip. **dmz**=dorsal marginal zone. **mhj**=midbrain/hindbrain junction. **pw**=posterior wall of the neuroenteric canal. **som**=somite. **tlb**=tailbud. **vmz**=ventral marginal zone.

All embryos are hybridised to a DIG labelled *eFGF* antisense probe. **A** is a vegetal view of an early gastrula stage 10.5 embryo (dorsal to the top). **B** is a vegetal view of a cleared early gastrula stage 10.5 embryo (dorsal to the top). **C** shows the cut surface of a stage 10.5 embryo that has been dissected along the dorsoventral midline (dorsal to the right). **D** is a vegetal view of a gastrula stage 11 embryo (dorsal to the top). **E** is a vegetal view of a gastrula stage 11.5 embryo (dorsal to the top). **F** shows the cut surface of a gastrula stage 11.5 embryo that has been dissected along the dorsoventral midline (dorsal to the right). **G** is a dorsal view of a late gastrula/early neurula stage 13 embryo (anterior is to the left). **H** shows the cut surface of a stage 13 embryo that has been dissected at right angles to the anteroposterior axis (transverse section) (dorsal to the top). **I** is a posterior view of a stage 13 embryo (dorsal to the top). **J** is a dorsal view of a neurula stage 14 embryo (anterior to the left). **K** is a posterior view of a cleared late neurula stage 20 embryo (dorsal to the top). **L** is a side view of the head region of a tailbud stage 32 embryo (anterior to the left). **M** is a side view of the head region of a cleared stage 32 embryo (anterior to the right, dorsal to the top). **N** is a side view of the posterior region of a stage 32 embryo (anterior to the left, dorsal to the top). **O** is a side view of the posterior region of a stage 32 embryo (anterior to the left, dorsal to the top).

Fig. 1. Whole-mount *in situ* hybridisation analysis of *Xenopus eFGF* expression during normal development.



However, yolky cells of the vegetal hemisphere contain far less mRNA than do the animal hemisphere cells. This means that, on a per unit volume basis, there is a predominantly animal localisation of the maternal *eFGF* pool. Unfortunately, using the techniques of radioactive and DIG *in situ* hybridisation it has not been possible to visualise the spatial distribution of *eFGF* mRNA during early cleavage or blastula stages. Using whole mount DIG *in situ* hybridisation *eFGF* expression is first detected following the onset of zygotic transcription in the early gastrula. At stage 10.5 *eFGF* mRNA is present in a complete ring around the blastopore, although the signal is considerably stronger on the dorsal side of the embryo (Fig. 1A, B and C). Staining for eFGF is stronger in the deep tissue layers and does not extend to the dorsal lip (Fig. 1C).

As gastrulation proceeds the levels of *eFGF* expression are maintained at similar levels in the lateral and ventral circumblastoporal regions and the highest level of expression is to be found in a 45 degree sector centred on the dorsal lip at mid-gastrula stage 11 and stage 11.5 (Fig. 1D and E). Thus the highest level of *eFGF* expression is in Spemann's organiser. In Fig. 1E *eFGF* expression is seen in the dorsal mesoderm after it has involuted and has begun to extend along the developing axis. This is more clearly shown in Fig. 1F, which is a cross section through a stage 11.5 embryo along the dorsoventral axis. The forming cavity of the archenteron is evident on the dorsal side of the embryo. At this stage *eFGF* is expressed in a posterior to anterior gradient within the involuting dorsal mesoderm. The most anterior extent of expression corresponds to the level of the anterior tip of the archenteron. Expression is absent from the endodermal lining of

the archenteron roof and the overlying ectoderm except at the dorsal lip, where staining can be seen in the superficial tissue layers as well as in the deep layers.

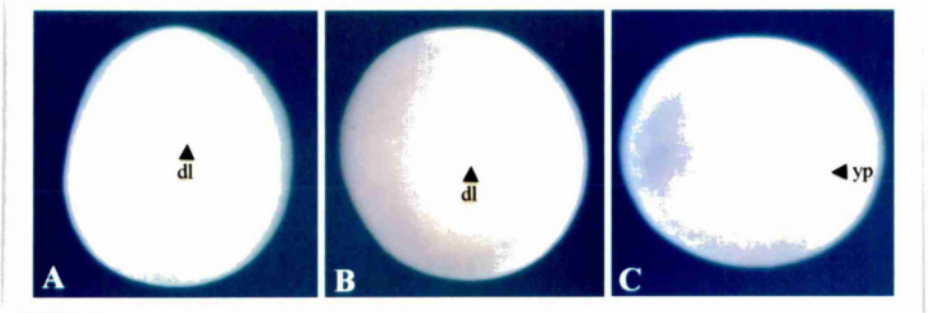
eFGF expression in the late gastrula and early neurula

Fig. 1G is a dorsal view of an embryo following closure of the blastopore at stage 13. The highest level of *eFGF* expression is still in the posterior of the embryo but staining can also be seen in the dorsal midline. Fig. 1H is a transverse section of a stage 13 embryo at a level 1/3 of the way along from the posterior tip, which shows that this staining is in the developing notochord. At this level in the embryo *eFGF* expression is not seen in the overlying neuroectoderm or underlying endoderm of the archenteron roof. In a number of specimens faint staining has been seen in the region of the somites that abuts the notochord.

Fig. 1I is a posterior view of a stage 13 embryo. Strong staining is seen in the dorsal midline and in wings of expression which spread laterally away from the closed blastopore. A sweep or "smile" of lower level expression can be seen ventrally below the blastopore. In contrast to the expression on the dorsal side of the blastopore, expression on the ventral side does not extend all the way to blastopore. On the dorsal and lateral aspects of the circumblastoporal region staining is seen in deep and superficial cells, although the superficial staining within the ectoderm appears to be somewhat pepper and salt in character. Ventrally the signal is restricted to the deeper cell layers.

The expression of *eFGF* along the whole length of the notochord is transient. Fig 1J. shows that by stage 14 the notochord staining is quite faint, whilst remaining strong in the posterior. This posterior restriction of expression

Fig. 2. Sense control whole-mount *eFGF in situ* hybridisations



Abbreviations: **dl**=dorsal blastopore lip. **yp**=yolk plug

All embryos are hybridised to a DIG labelled *eFGF* sense probe. **A** is a vegetal view of a gastrula stage 11 embryo (dorsal to the top). **B** is a vegetal view of a gastrula stage 12 embryo (dorsal to the top). **C** is a dorsal view of a late gastrula stage 13 embryo (anterior to the left).

XFGF-3

FGF-3 expression in the early gastrula

Unlike *eFGF* which has a low level of maternal expression, *Xenopus FGF-3* is only expressed zygotically. However, like *eFGF*, *FGF-3* is most highly expressed in the gastrula and neurula but the level of *FGF-3* expression does not fall off as dramatically as *eFGF* during subsequent development and is maintained at a more constant level up to the swimming larva stage 40 (Tannahill *et al.*, 1992).

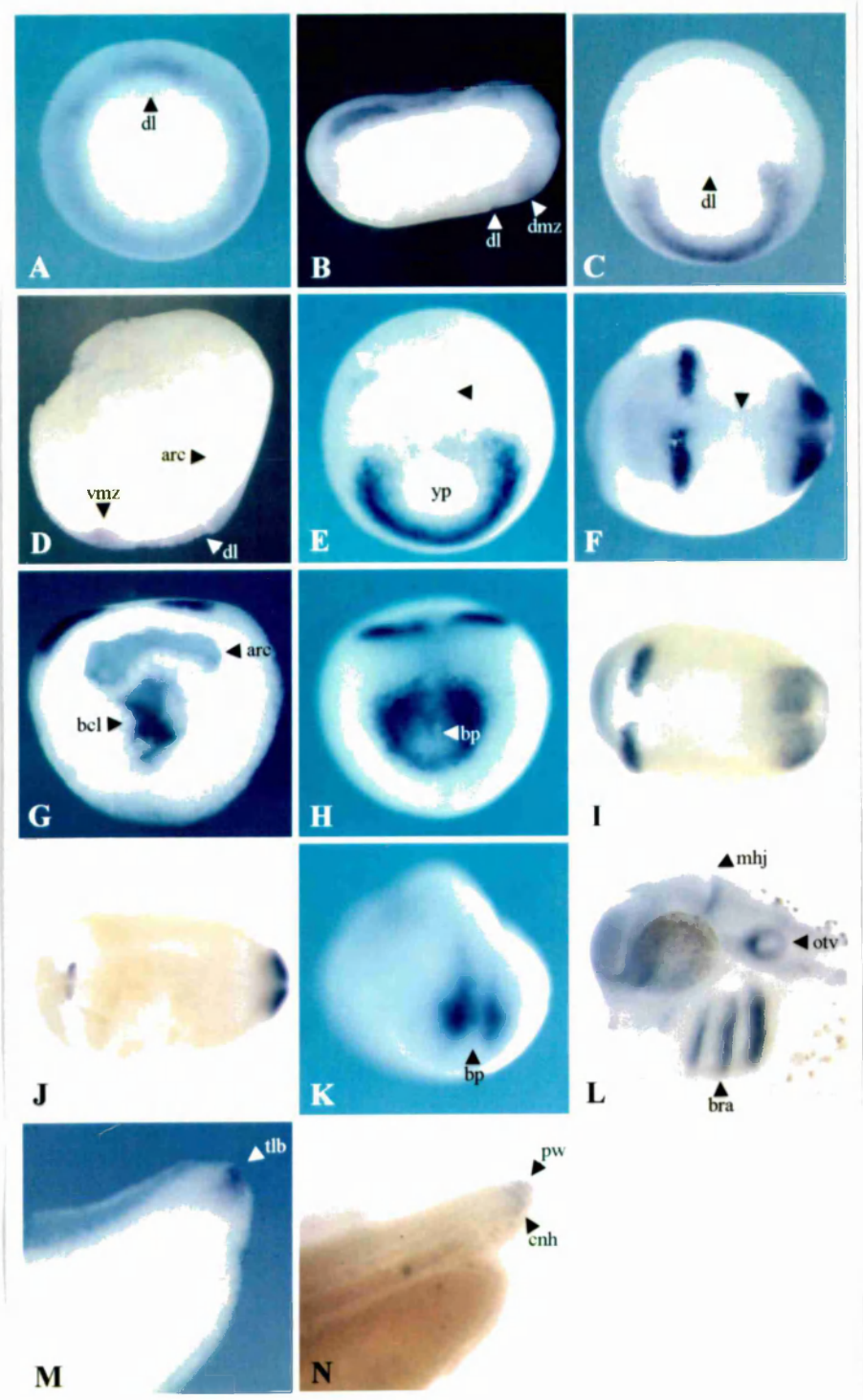
Using whole-mount DIG *in situ* hybridisation expression is first detected at the early gastrula stage 10.5 and is apparent as a faint staining in a complete ring around the blastopore (Fig. 3A). At this stage the expression of *FGF-3* is very similar to that of *eFGF* and, just as with *eFGF*, there is a higher level of expression seen on the dorsal side of the embryo (Fig 3B). However, unlike *eFGF*, as gastrulation proceeds *FGF-3* expression becomes somewhat down regulated on the dorsal side of the embryo relative to the lateral and ventral aspects of the blastopore (Fig. 3C). Also, the staining seen with *FGF-3* seems more restricted to the superficial layers than is the case with *eFGF* (Fig. 3D). Later in gastrulation another domain of *FGF-3* expression is visible as single stripes in the ectoderm either side of the dorsal midline about halfway along the embryo length. These two stripes of expression are linked by very faint streaky expression in the ectoderm either side of the dorsal midline (Fig. 3E). It is important to note that unlike *eFGF*, *FGF-3* expression is not detected in the developing notochord.

Fig. 3. Whole-mount *in situ* hybridisation analysis of *Xenopus FGF-3* (*int-2*) expression during normal development.

Abbreviations: **arc**=archenteron. **bcl**= blastocoel. **bp**=blastopore. **bra**=branchial arches. **cnh**=chordoneural hinge. **dl**=dorsal blastopore lip. **dmz**=dorsal marginal zone. **mhj**=midbrain/hindbrain junction. **otv**=otic vesicle. **pw**=posterior wall of the neuroenteric canal. **som**=somite. **tlb**=tailbud. **vmz**=ventral marginal zone.

All embryos are hybridised to a DIG labelled *FGF-3* (*int-2*) antisense probe. **A** is a vegetal view of an early gastrula stage 10.5 embryo (dorsal to the top). **B** shows the cut surface of a stage 10.5 embryo that has been dissected along the dorsoventral midline (dorsal to the right). **C** is a dorsovegetal view of a gastrula stage 11.5 embryo (dorsal to the top). **D** shows the cut surface of a stage 11.5 embryo that has been dissected along the dorsoventral midline (dorsal to the right). **E** is a dorsovegetal view of a late gastrula stage 12.5 embryo. Black arrow indicates faint ectodermal midline staining. White arrow indicates anterior ectodermal stripe of expression (dorsoanterior to the top). **F** is a dorsal view of late gastrula/early neurula stage 13 embryo (anterior is to the left). Black arrow indicates faint ectodermal midline staining. **G** shows the cut surface of a stage 13 embryo that has been dissected at right angles to the anteroposterior axis at the level of the *FGF-3* anterior ectodermal stripe of expression (transverse section) (dorsal to the top, anterior into the page). **H** is a posterior view of a stage 13 embryo (dorsal to the top). **I** is a dorsal view of a cleared neurula stage 14 embryo (anterior to the left). **J** is a dorsal view of a cleared late neurula stage 20 embryo (anterior to the left). **K** is a posterior view of a stage 20 embryo (dorsal to the top). **L** is a side view of the head region of a cleared tailbud stage 32 embryo (anterior to the right, dorsal to the top). Black arrow indicates the otic vesicle. **M** is a side view of the posterior region of a stage 32 embryo (anterior to the left, dorsal to the top). **N** is a side view of the posterior region of a cleared stage 32 embryo (anterior to the left, dorsal to the top).

Fig. 3. Whole-mount *in situ* hybridisation analysis of *Xenopus FGF-3* (*int-2*) expression during normal development



Volume 2

The Fibroblast Growth Factor Family in the Early Development of *Xenopus laevis*.

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FGF-3 expression in the late gastrula stage and early neurula

Fig. 3F is dorsal view of a stage 13 embryo following the closure of the blastopore and shows that *FGF-3* expression is highly expressed in the posterior of the embryo around the closed blastopore. The level of expression of *FGF-3* is still somewhat reduced in the dorsal midline of the posterior, although there is still some faint staining in the superficial layers either side of the midline running up to the level of the prominent anterior stripes of expression. A comparison with the fate map of Eagleson and Harris, (1990) suggests that at this stage the cells from the anterior domain of *FGF-3* expression will contribute to both midbrain and hindbrain. Fig. 3G shows that the anterior stripes of expression are restricted to the ectoderm of the embryo. Fig. 3H is a posterior view of a stage 13 embryo that shows *FGF-3* expression around the closed blastopore. Note in common with *eFGF* there is a ventral "smile" of expression that does not go right up to the blastopore. Fig. 3I is a cleared early neurula stage 14 embryo, and shows that *FGF-3* expression is absent from the developing notochord.

The dorsal view of a cleared stage 20 embryo at the end of the neurula stage (Fig. 3J) shows that the anterior stripes of expression are now much narrower and closure of the neural folds has brought the anterior stripes of *FGF-3* expression close together so that they are almost touching in the dorsal mid-line. A comparison of the expression of *FGF-3* with that of *krox20*, which is expressed in rhombomeres 3 and 5, (Bradley *et al.*, 1992) and *en-2* (Hemmati-Brivanlou and Harland, 1989), which is a marker of the midbrain/hindbrain junction (data not shown) suggests that at this stage the *FGF-3* stripe lies within the hindbrain, at about the level of rhombomere 5. A posterior view of an embryo from stage 20

(Fig. 3K) shows strong *FGF-3* expression in regions lateral to the closed blastopore. This expression extends for some distance along the developing axis but expression remains absent from the dorsal midline.

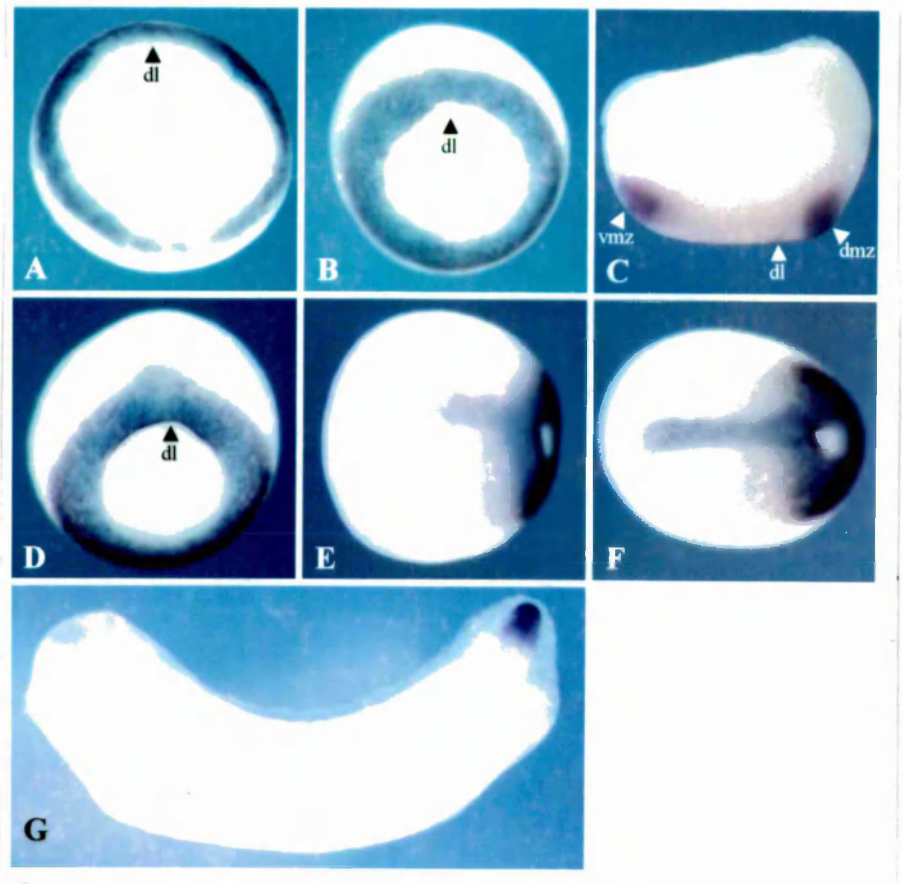
FGF-3 expression during later development

Fig. 3L is a cleared specimen of a tailbud embryo stage 31 and shows the expression of *FGF-3* within the developing head. High levels of expression can be seen in the lining of all three pharyngeal pouches and in the region of the otic vesicle. There is also a prominent stripe of expression anterior to the otic vesicle at the level of the midbrain/hindbrain junction. It is likely that this represents a *de novo* site of expression and is not an elaboration of the hindbrain expression seen during neurula stages. In the posterior of the embryo Fig. 3M and Fig. 3N show that *FGF-3* is expressed in the developing tailbud in both the chordoneural hinge and the posterior wall of the neuroenteric canal.

Xbra

The expression pattern of *Xbra* has been widely reported elsewhere (Smith *et al.*, 1991; Ruiz i Altaba and Jessell, 1992; Isaacs *et al.*, 1995; Vodicka and Gerhart, 1995). However, an examination of the close relationship between certain of the *FGFs* and *Xbra* is a major theme of this thesis. It was therefore decided to include a number of *in situs* to aid in the comparison of expression patterns of the *FGFs* and *Xbra*.

Fig. 4. Whole-mount *in situ* hybridisation analysis of *Xenopus* *Xbra* expression during normal development.



Abbreviations: **dl**=dorsal blastopore lip. **dmz**=dorsal marginal zone. **vmz**=ventral marginal zone.

All embryos are hybridised to a DIG labelled *Xbra* antisense probe. **A** is a vegetal view of a very early gastrula stage 10 embryo. **B** is a vegetal view of a gastrula stage 10.5 embryo. **C** shows the cut surface of a stage 10.5 embryo that has been dissected along the dorsoventral midline (dorsal to the right). **D** is a dorsovegetal view of a gastrula stage 11 embryo (dorsal to the top). **E** is a dorsal view of a late gastrula stage 13 embryo (anterior to the left). **F** is a dorsal view of a neurula stage 14 embryo (anterior to the left). **G** is a side view of a tailbud stage 32 embryo (anterior to the left, dorsal to the top).

Xbra expression during development

In the very early gastrula stage 10 *Xbra* expression is detected in a complete ring around the forming blastopore and, as is the case with *eFGF* , this expression is in a dorsal to ventral gradient (Fig. 4A). By gastrula stage 10.5 the levels of *Xbra* expression have risen considerably and a dorsal to ventral gradient is less apparent, although is possible that a gradient pattern is obscured due to the high level of expression which may lead to saturated staining (Fig. 4B). The stage 10.5 embryo in Fig. 4C has been cut open along the future dorsoventral axis and shows strong *Xbra* staining within the dorsal and ventral marginal zone. On the dorsal side of the embryo *Xbra* expression is seen within both the deep and superficial tissue layers. The superficial tissue layer is fated to form the lining of the archenteron roof and indicates that *Xbra* expression is found not only in mesoderm but also within endodermal precursors. At this stage *Xbra* expression is not present in the deepest cell layers and does not yet extend all the way to the blastopore lip. This non-expressing population of cells corresponds to the anterior mesendodermal cells which contribute to the mesoderm of the head and the pharyngeal endoderm. It is this cell population which as been shown to express the transcription factor *goosecoid* and it is significant that there is no appreciable overlap of *goosecoid* and *Xbra* expression domains (Vodicka and Gerhart, 1995).

By mid-gastrula stage 11 (Fig. 4D) *Xbra* expression extends all the way to the dorsal blastopore lip and expression can be seen in the involuting dorsal mesoderm as it begins to extend along the developing anteroposterior axis. By the end of gastrulation (Fig. 4E and F) *Xbra* expression is seen in the whole length of the notochord and in a ring around the closed blastopore in a pattern that is

very reminiscent of the *eFGF* expression pattern. At this stage Vodicka and Gerhart, (1995) report that *Xbra* is expressed in both deep and superficial layers in the posterior. In later development *Xbra* expression is lost from the mature notochord but continues to be expressed in the same regions of the tailbud as *eFGF* (Fig. 4G)

Discussion

The expression patterns of eFGF, FGF-4 and FGF-6

The predicted amino acid sequence of Xenopus eFGF is equally related to that of mammalian FGF-4 and FGF-6 (Isaacs *et al.*, 1992). The exact nature of the evolutionary relationship of these molecules is at present unclear, however a number of possibilities present themselves. Firstly, it is possible that eFGF does in fact represent a direct homologue of *FGF-4* or *FGF-6*. However, simple sequence analysis is not conclusive in this matter. Alternatively, it is possible that *eFGF* represents a gene that is ancestral to both *FGF-4* and *FGF-6* that underwent a duplication event following the divergence of the amphibian and mammalian lines. A third possibility is that eFGF actually represents a new member of the FGF family with an as yet undiscovered direct homologue in the higher vertebrates. This is supported by the fact that eFGF only shares 60% amino acid identity with its putative mammalian homologues FGF-4/FGF-6. This is somewhat lower than the other *Xenopus* FGFs to their mammalian homologues (bFGF=83%, FGF-3=71%, FGF9=93%). This means that the identity between eFGF and FGF-4/FGF-6 (60%) is less than the identity between FGF-4 and FGF-

6 (70%). Certain aspects of the expression pattern of *eFGF* are also suggestive that the latter proposal might be the case.

Judging from expression pattern alone *eFGF* is more closely related to *FGF-4* than *FGF-6.* The expression of *FGF-6* is restricted to the developing muscle lineages and in the mouse expression is not detected until quite late in development (day 9.5 post-conceptus) (De Lapeyriere *et al.*, 1993). *FGF-4* is expressed in the embryonic ectoderm before the start of gastrulation and its expression then becomes localised to the migrating mesoderm cells within the primitive streak and later in the tailbud region (Niswander and Martin, 1992; Drucker and Goldfarb, 1993). This is directly comparable to the early expression of *eFGF* in the animal hemisphere during blastula stages and within the mesoderm around the blastopore during gastrulation and later within the tailbud (Isaacs *et al.*, 1992). However, there are significant differences, most importantly *FGF-4* expression has not been detected in the notochord. *eFGF* is the only member of the FGF family which has been shown to be expressed in the developing notochord. Furthermore, unlike *eFGF*, *FGF-4* expression is not detected within the developing central nervous system at the midbrain/hindbrain junction. These data provide some support for the view that there is a direct homologue of eFGF in the higher vertebrates that is distinct from FGF-4 and FGF-6.

The expression of FGF-3 in Xenopus and higher vertebrates

The initial expression of mouse *FGF-3 is* detected in the extraembryonic parietal endoderm. However, the first expression that is detected in the embryo proper is

within the early migrating mesoderm of the primitive streak (Wilkinson *et al.*, 1988). This is similar to the onset of *FGF-3* expression within the mesoderm of the periblastoporal region in *Xenopus* and recently reported findings that show *FGF-3* expression in the early primitive streak of the chick (Tannahill *et al.*, 1992; Mahmood *et al.*, 1995b). Similarly, in both *Xenopus* and chick *FGF-3* is not expressed within the developing notochord but is expressed at low levels in the dorsal midline ectoderm, possibly in cells which will later contribute to the floor plate of the neural tube.

In mouse *FGF-3* expression is detected in the region of rhombomeres 4 and 5 (r4 and r5) of the hindbrain adjacent to the developing otocyst (Wilkinson *et al.*, 1988). As expression within the hindbrain falls, expression within the developing otocyst increases. This apparent close relationship between *FGF-3* expression and the development of the otocyst has led to much speculation that FGF-3 may be involved in the induction of the otic vesicle (Wilkinson *et al.*, 1988; Represa *et al.*, 1991). Recent data obtained from mouse null mutants for *FGF-3* cast doubt on this notion (Mansour *et al.*, 1993; Mansour, 1994). These studies indicate that *FGF-3* is not required for the induction of the otocyst but is required for the subsequent development of the ear. In chick and *Xenopus* the expression of *FGF-3* within the central nervous system (CNS) is complex and dynamic. In the chick *FGF-3* the initial expression within the CNS is detected as two patches either side of the dorsal midline in a region of the unsegmented prospective hindbrain. When rhombomere boundaries are first apparent expression becomes restricted to r4 and r5 and then r6. Finally its expression becomes restricted to rhombomere boundaries. The initial neural expression domain of *FGF-3* in

Xenopus is rather broader than is the case in chick or mouse and probably fate maps to regions of prospective midbrain and hindbrain. However, in the early tailbud expression is restricted to the region of r5. Later in development expression of *Xenopus FGF-3* is not detected within the rhombomeres but a new domain of expression is seen at the midbrain/hindbrain junction. This domain of expression is also detected in the chick.

During later development the expression pattern of *FGF-3* becomes increasingly complex, particularly in the head, and makes the comparison of expression in different species difficult. However, certain features are conserved, for example, in all 3 species examined *FGF-3* continues to be expressed in the posterior of the embryo in the tailbud region and in the anterior is expressed in the pharyngeal pouches. In *Xenopus* and mouse *FGF-3* is expressed in the developing eye and in *Xenopus* and chick expression is found in the region where the stomodeal opening will form.

Brachyury in development

Brachyury homologues in the mouse (*T*), chick (*CH-T*), zebrafish (*no tail, ntl*) and *Xenopus* (*Xbra*) not only share a high degree of amino acid sequence identity (78-91%) but also have very similar patterns of expression. The details of *Xbra* expression in *Xenopus* are directly comparable to the expression of *Brachyury* within the developing mesoderm and endoderm of other vertebrates (Wilkinson *et al.*, 1990; Smith *et al.*, 1991; SchulteMerker *et al.*, 1994c; Kispert *et al.*, 1995b)

In *Xenopus* there is a very low level of maternal *Xbra* expression and zygotic expression is first detected soon after the mid-blastula transition (MBT)

(Smith *et al.*, 1991). At the start of gastrulation *Xbra* is expressed in the marginal zone of the embryo including the most superficial layer of cells which forms the endodermal lining of the archenteron. However, expression of *Xbra* is absent from the very deepest layers of cells which contribute to the head mesoderm and pharyngeal endoderm. As gastrulation proceeds, and cells of the mesoderm involute, expression is rapidly down regulated in all cells except the notochord and cells around the closed blastopore, where expression persists through neurula stages. Expression of *Xbra* becomes increasingly localised to the posterior of the embryo and is found in the tailbud during tail extension.

The phenotype of the '*T*' mutant mice and *no tail* mutant zebrafish is also strikingly similar. In both naturally occurring mutations anterior development is rather normal. However, in *no tail* zebrafish trunk structures posterior of somite 11-13 are lost (SchulteMerker *et al.*, 1994c) and in homozygous T mice development of the trunk and tail posterior to somite number 7 is lost (Beddington *et al.*, 1992). Furthermore in both mutants there is a failure of the notochord to differentiate. In mouse there is also abnormal development of the allantois, which contributes to the placenta. This leads to reduced supply of nutrition to the embryo and ultimately to the lethality of this mutation at mid-gestation.

From these data it is apparent that there is a high degree of conservation of both expression and function for *Brachyury* and furthermore suggest that *Brachyury* is likely to play similar roles in the development of the notochord and posterior mesoderm in all vertebrates. Additional support for this view is provided by data in *Xenopus* that shows that the overexpression of *Xbra* in animal cap

explants results in the formation of mesodermal tissue types (Cunliffe and Smith, 1992).

There is further recent evidence that the role of *Brachyury* in the development of the mesoderm, and in particular the notochord, may extend to the primitive chordate ancestors of the vertebrates. *Brachyury* homologues (*As-T* and *As-T2*) have been cloned from the urochordate ascidian *Halocynthia roretzi* (Yasuo and Satoh, 1994). Interestingly *As-T* is only expressed in notochord and not in the mesenchyme. *As-T2*, on the other hand, is expressed in the involuting muscle cells of the gastrula and the tail tip of the neurula (Yasuo *et al.*, 1995). This may suggest that the function of *Brachyury* is split between 2 genes in the ascidians. However, the sequence of the *As-T2* 'T-box' domain is divergent from other chordate *Brachyury* homologues and (Yasuo *et al.*, 1995) indicates that the expression of *As-T* exclusively in the notochord represents the expression domain of the ancestral *Brachyury* gene. The expression of pattern of two *Brachyury* homologues (*AmBra*) in the cephalochordate amphioxus (*Branchiostoma floridae*) is remarkably similar to that of the vertebrates and expression is detected in both the notochord and the mesoderm around the closing blastopore (Holland *et al.*, 1995). The presence of homologues in the 3 chordate sub-phyla (vertebrates, cephalochordates and urochordates) argues that the function of *Brachyury* in the formation of the mesoderm is very ancient.

Brachyury and the FGFs

Further details of the close relationship between the regulation of *Xbra* expression, its activities and FGF signalling will be discussed in subsequent

chapters. However, data in this chapter show that there is considerable overlap of expression between *Xbra* and the known secreted *Xenopus* FGFs, in particular *eFGF*. During gastrula and neurula stages *eFGF* and *Xbra* are expressed in very similar domains in the developing mesoderm including the notochord. During later development both are localised to the chordoneural hinge and posterior wall of the neuroenteric canal of the tailbud (Gont and De Robertis, 1993; Isaacs *et al.*, 1995; Tucker and Slack, 1995). There is less overlap of expression between *Xbra* and *FGF-3*, however, both genes are expressed early in the periblastoporal region and later in the tailbud.

The other known *Xenopus* FGFs, *bFGF* and *FGF-9,* do not have highly restricted patterns of expression and are much more widely expressed during development (Song and Slack, 1994; Song and Slack, in press). At no stage do *bFGF* and *FGF-9* share the tight co-localisation of expression with *Xbra* that is the case with *eFGF* and *FGF-3*.

In higher vertebrates, again it can be seen that a number of the FGFs have expression patterns which overlap that of *Brachyury*. In the mouse, *FGF-3*, *FGF-4* and *FGF-8* expression is found within the nascent mesoderm of the primitive streak during gastrulation and later in the tailbud (Wilkinson *et al.*, 1988; Niswander and Martin, 1992; Crossley and Martin, 1995). Significantly, however, no mammalian FGF has yet been shown to be expressed within the cells of the notochord.

FGFs and mesoderm induction

Much of the background to the study of the function of the FGFs in *Xenopus* development is influenced by the observation that these factors have potent mesoderm inducing activity and have been considered as candidates for one of the *in vivo,* maternal, vegetally localised mesoderm inducing factors. This now seems unlikely because the available data shows that the maternal FGFs are not localised to the vegetal hemisphere. Furthermore, the zygotic expression of *eFGF* and *FGF-3* within the nascent mesoderm during gastrula stages indicates that they have roles during the subsequent development of the mesoderm.

The FGF receptors in Xenopus

Three members of the receptor family have been cloned in *Xenopus*. Studies of the distribution of *FGF-R1* mRNA indicate that it is present thoughout the early stages of development (Musci *et al.*, 1990). As with the FGF ligand mRNA, on a per unit volume basis, *FGF-R1* mRNA is more abundant in the marginal zone and animal hemisphere. Western blot data confirm that the receptor protein also has a predominantly animal localisation (Cornell *et al.*, 1995). In the late gastrula stage immunohistochemical data shows that the receptor is most abundant in the blastopore region (Ding *et al.*, 1992). *FGF-R2* is not expressed in the blastula but is first detected in the anterior neural plate during gastrulation (Freisel and Brown, 1992). The expression of FGF-R2 suggests that it is not involved in the establishment of the mesoderm. However, *FGF-3* has an anterior domain of expression within the ectoderm of the gastrula and it is possible that FGF-R2 is involved in transducing the FGF-3 signal. This idea is further supported by recent

evidence that shows that *Xenopus* FGF-3 protein can bind with high affinity to the IIIb and IIIc isoforms of FGF-R2 (Mathieu *et al.*, 1995). *FGF-R4* has also recently been cloned in *Xenopus* and is expressed throughout early development. At present there is no report of its localisation (Shiozaki *et al.*, 1995).

The whole-mount *in situ* study in this chapter confirms previous findings that, both during gastrula and neurula stages, there is considerable overlap in the expression patterns of the *eFGF*, *FGF-3* and *Xbra* within the newly formed mesoderm. However, it unexpectedly reveals that later in gastrulation *eFGF* is most highly expressed in the dorsal mesoderm and continues to be expressed within the developing notochord during the neurula stage. In contrast, the expression of *FGF-3* becomes somewhat down regulated within the dorsal mesoderm as gastrulation proceeds and is not expressed within the notochord. These data demonstrate a very close correlation between the expression patterns of *eFGF* and *Xbra* during early development. Moreover, the expression pattern of *FGF-3* suggests that it likely to have roles in common with *eFGF*, particularly in the posterior, but also has distinct roles during anterior development.

Chapter 4

Mesoderm induction by eFGF.

Introduction

The mesoderm of amphibians arises in a ring around the equator of the embryo in the so-called marginal zone (MZ). If explants taken from the prospective MZ of the newt *Triturus* prior to the 64-cell stage are cultured in isolation they form only epidermis. If they are taken at a later stage they will form both epidermis and mesoderm (reviewed by Smith, 1989). This suggests that during normal development the formation of mesoderm in the MZ requires the presence of factors which are absent from the explant before the 64-cell stage. Such a difference between what a group of cells forms in isolation (specification) and what they give rise to in normal development (fate) indicates that an inductive process is involved in their normal development. Induction can be defined as any process in development which involves a group of cells in one part of the embryo producing a signal that can redirect the developmental fate of another group of cells within the embryo.

The work of Nieuwkoop in the axolotl (Nieuwkoop, 1969) and *Xenopus* (Sudarwati and Nieuwkoop, 1971) indicates that the source of the mesoderm induction signal is the yolky cells of the vegetal hemisphere. If explants taken from the animal pole region and the vegetal pole region are cultured in isolation they form only epidermis and endoderm respectively. If however, they are cultured in combination mesodermal tissue forms as well. The use of lineage labels demonstrates that the mesoderm in such combinations arises from the animal pole tissue (Sudarwati and Nieuwkoop, 1971; Dale *et al.*, 1985). This indicates that the source of the mesoderm inducing signal is the vegetal hemisphere and further demonstrates that it is not only the cells of the MZ which

can respond to these signals but that the whole of the animal hemisphere is competent to respond.

Experiments of this sort have further shown that the vegetal hemisphere can be divided into 2 regions based upon the type of mesoderm that it induces in such 'Nieuwkoop' combinations (Boterenbrood and Nieuwkoop, 1973; Dale and Slack, 1987b). Most of the vegetal hemisphere will induce extreme ventral-type mesoderm such as blood, mesothelium and loosely packed mesenchyme and is termed the ventrovegetal (VV) region. A rather smaller region of the vegetal hemisphere has the ability to induce dorsal-type mesoderm such as notochord and muscle and is termed the dorsovegetal (DV) region or the 'Nieuwkoop centre' as it has been called in honour of the great man. There also appears to be some dorsoventral prepattern in the animal hemisphere so that animal hemisphere explants from the dorsal side are more likely to form dorsal structures in response to a given factor than is a similar explant from the ventral half of the animal hemisphere (Sokol and Melton, 1991).

The exact nature of how the dorsoventral axis is established in *Xenopus* is something of a mystery. Within the first 45 minutes following fertilisation there is a rotation of the cortical cytoplasm relative to the deep cytoplasm towards the site of sperm entry. The direction of this rotation is towards the future ventral side away from the future dorsal side of the embryo (Gerhart *et al.*, 1989). Thus the dorsovegetal signalling centre, and the dorsal region of the animal hemisphere with a dorsal bias in response to inducing factors, is set up in the region of the embryo where there is a net movement of vegetal tissue into the animal hemisphere. At the moment the mechanism by which the dorsal signalling and

responding properties are established in this shear zone is unclear but it must involve some activation, by translational or post-translational modification, of pre-existing maternal components on the dorsal side. The presence of an exclusive maternal dorsal determinant is unlikely because prior to fertilisation eggs are radially symmetrical and the future dorsoventral axis can be established along any of the radial planes of symmetry by natural or artificial stimulation of the cortical rotation (Gerhart *et al.*, 1989). Therefore the components necessary for the formation of the dorsal signalling centre are also likely to be distributed in a radially symmetrical fashion.

The use of heterochronic animal and vegetal 'Nieuwkoop' combinations has established that mesoderm induction probably begins during the early blastula stage before the onset of zygotic transcription at the midblastula transition (MBT) (Jones and Woodland, 1987). This is an important consideration because it means that at least the initial components necessary for mesoderm induction must be present maternally. The competence of the animal hemisphere to respond to the vegetal signals is lost at the start of gastrulation. At this stage the specification of the marginal zone closely reflects the size and inducing qualities of the underlying vegetal tissue (Dale and Slack, 1987b). However, the dorsoventral specification of the MZ at this stage is quite different from the normal fate map projection, indicating that further interactions take place within the mesoderm during gastrula stages. For example, during gastrula stages a signal from the dorsal mesoderm results in the ventrally specified regions of the MZ adopting a more lateral character. The processes has become known as 'dorsalisation' and

has received considerable attention in recent years (Smith and Slack, 1983; Dale and Slack, 1987b; Lettice and Slack, 1993).

An important development of "Nieuwkoop combinations" has been the animal cap serial dilution assay. Using this assay the mesoderm inducing activity of a given factor can be quantified. Briefly, mid-blastula stage animal cap explants are exposed to serial dilutions of the factor and cultured for up 3 days after which they are analysed histologically or with molecular markers for mesoderm formation. The animal cap assay has also been extended to test the autoinducing activity of injected mRNAs, where it is assumed that the effective quantities of active proteins produced is proportional to the injected amounts of mRNA. It is the development of this assay system which in recent years has allowed a major step forward in the understanding of the process of mesoderm induction and importantly the identification of purified factors capable of mimicking the endogenous mesoderm inducing signals.

Much work has concentrated on the inducing activities of the fibroblast growth factors (FGF) and members of the transforming growth factor ß (TGFß) family, in particular the activins and bone morphogenetic proteins (BMP). The demonstration that FGFs induce ventral-type mesoderm and the activins induce dorsal-type mesoderm in the animal cap assay quickly established them as candidates for the endogenous inducing signals. This candidacy was further strengthened when members of both families were shown to be present in the early embryo (reviewed by Kessler and Melton, 1994; Slack, 1994). However, as knowledge of these molecules has increased the views on their functions in development have been modified. The complete absence of mesoderm in

embryos in which the activin signal transduction pathway is inhibited (Hemmati-Brivanlou and Melton, 1992), and the recent demonstration that an activin responsive reporter construct is activated in the whole of the vegetal hemisphere still indicate that an activin-like molecule is part of the vegetal inducer (Watabe *et al.*, 1995). Expression data however, indicate that this is unlikely to be activin itself (Dohrmann *et al.*, 1993). The best candidate at present is vg1, which has the requisite vegetal localisation and has now been shown to have mesoderm inducing activity (Weeks and Melton, 1987; Dale *et al.*, 1993; Thomsen and Melton, 1993). In this view however, vg1 represents a general mesoderm inducing signal that is active throughout the vegetal hemisphere in both the VV and DV regions. The DV inducing signal is qualitatively different from the VV signal, as judged by its ability to induce mesoderm with the properties of the organiser. Evidence from overexpression of members of the wnt secreted molecule family and overexpression or inhibition of down stream elements of the wnt signal transduction pathway support the view that signalling from a member of the wnt family contributes to the DV signal from the 'Nieuwkoop centre' (Sokol *et al.*, 1991; Heasman *et al.*, 1994; He *et al.*, 1995).

The interactions between the various candidate signalling molecules is extremely complex, and studies of these interactions have provided evidence that the secreted molecules noggin, chordin and Xnr form the molecular basis of dorsalisation (Smith *et al.*, 1993; Holley *et al.*, 1995; Jones *et al.*, 1995). Dorsalisation as a process was identified by cut and paste embryology but the dawn of the molecular era has provided insights into processes which were unsuspected from classical embryology. For example, BMP4 has a potent ventral

mesoderm inducing activity on animal cap explants (Dale *et al.*, 1992; Jones *et al.*, 1992), but the best data at present has it playing an important role during gastrulation as a ventralising signal that counters the dorsalising influence of the organiser (Maeno *et al.*, 1994; Hawley *et al.*, 1995; Ishikawa *et al.*, 1995; Suzuki *et al.*, 1995; Jones *et al.*, 1996). In this way competing dorsal and ventral signals establish the relative sizes of the dorsal and ventral mesodermal territories during gastrulation.

Similarly the role that is emerging for the FGFs is quite different from that originally envisaged. Maternal expression of the known *Xenopus* FGFs is predominately to the animal rather than the vegetal hemisphere making them unlikely candidates for the vegetal inducer (this thesis; Song and Slack, 1994). However, it is the demonstration that the FGFs have potent mesoderm inducing activity in the animal cap assay that has paved the way for the understanding of what the FGFs do during normal development. This chapter examines the properties of eFGF as a mesoderm inducing factor in a number of animal cap based assays. These data show that eFGF is a secreted factor with potent mesoderm inducing activity and that the type of mesoderm that it induces, in common with other FGFs, is of a ventrolateral character.

Materials and methods

Embryo injections and manipulations

Embryos were prepared and cultured as per general methods. Capped synthetic mRNAs for injection were transcribed as per general methods. The pSP64-eFGF plasmid is as described by Isaacs *et al.*, (1994) and contains the naturally

occurring sequence around the initiator codon. The pSP64-XbFGF plasmid is as described by Thompson and Slack, (1992) and also contains the natural initiator sequence. Embryos for animal caps were injected into both blastomeres in the animal pole region following first cleavage. Animal caps were taken at stage 9 as per general methods and cultured until the required control stage. For the Einsteck-procedures animal caps from FDA injected embryos were explanted at stage 8 and treated with 100 ng/ml eFGF. At control stage 10 animal caps were grafted into the blastocoel of unlabeled hosts as per general methods.

Production of recombinant eFGF protein.

Recombinant eFGF protein used in this study was produced as part of a collaborative project in the laboratory of Arnold Coffer (ICRF, Lincoln's Inn Fields) using the pET T7 expression system (Rosenberg *et al.*, 1987) and a modification of the methods of Isaacs *et al.*, (1992). Briefly, a NcoI/BamHI fragment of XeFGF(i) was cloned into the pET8c translation vector. Cutting with Nco I has the effect of truncating the protein by 44 amino acids at its amino terminus and has previously been shown to be biologically active in the mesoderm inducing assay (Isaacs *et al.*, 1992).

BL21(DE3) pLys S bacteria transformed with the XeFGF(i)-pET construct, are grown in liquid culture (L. broth + chloramphenicol 25 mg/ml +ampicillin 200 mg/ml) with vigorous shaking at 37^0 C until $O.D_{600}$ reaches 0.6. Protein production is induced following the methods of (Rosenberg *et al.*, 1987) by the addition of IPTG to 0.4 mM. The bacteria are pelleted and resuspended in a small volume of ice cold 50 mM tris pH 8.0 +1 mM EDTA + 1 mM PMSF + 0.1

mg/ml pepstatin A and then lysed by sonication on ice. Bacterial debris is removed by ultracentrifugation. The clear supernatant is then loaded onto a heparin sepharose column and washed with 50 mM tris pH 8.0 +1 mM EDTA + 0.5 M NaCl. The column is eluted with a 0.5 M to 2.0 NaCl gradient at 0.5 ml/ minute over a 30 minute period collecting 1 minute fractions. Fractions from around the O.D. peak, giving a single band as determined by SDS-PAGE electrophoresis, are pooled. Glycerol is added to 50 % and aliquots frozen down at -70^0 C. Yields of up to 10 mg of purified eFGF protein/ litre of bacterial culture can be prepared in this way.

In vitro translation of proteins

Synthetic *eFGF (i)* mRNA was transcribed as per general methods using the pSP64-eFGF (mod) plasmid (Isaacs *et al.*, 1992). The sequence around the initiator ATG of this construct has been modified using a PCR based approach to optimise translation *in vitro* and *in vivo*. The synthetic *eFGF* mRNA was translated *in vitro* along with relevant control mRNAs. Canine pancreatic microsomes were included to test core post-translational processing of radioactivity labelled synthetic proteins.

1 to 3 µg of heat denatured mRNA was added to a 25 to 50 µl mixture containing 70% rabbit reticulocyte lysate (Promega), 1 mM amino acids (-methionine, (Promega)) and 1 µCi/µl of labelling grade ^{35}S-methionine (Amersham). Canine microsomes (Promega) were added at 1 µl/50 µl of reaction mixture. The reactions were incubated at 30^0 C for 50 minutes and then 5 µl of the products were analysed by SDS-PAGE and autofluorography.

Polyacrylamide gel electrophoresis

SDS-PAGE gels were run using standard protocols (Sambrook *et al.*, 1989). Protein gels were fixed in 40% methanol and 10% acetic acid and impregnated with fluors using Amplify (Amersham) as per manufactures instructions. Gels were dried down on to filter paper and autofluorography was performed using XAR X-Omat film (Kodak).

Histology

Carried out as per general methods

RNAase protection analysis

As per general methods. The expression of *ornithine decarboxylase* (*ODC*) gene was used as an internal loading control (Isaacs *et al.*, 1992). *Xbra* was detected as in Smith *et al.*, (1991). *eFGF* as in Isaacs *et al.*, (1992). *Noggin* expression was deteteced as in Isaacs *et al.*, (1992). *Xwnt-8* expression was detected as in Christian and Moon, (1993).

Results

The secreted nature of eFGF

Sequence analysis eFGF (i) using the rules of von Heijne, (1986) predicts that eFGF is a secreted protein with a cleavable signal peptide and that cleavage is likely to occur at either position 23 or 29 (Isaacs *et al.*, 1992). In addition the sequence Asn.Asp.Thr (amino acids 30.31.32) conforms to the consensus N-

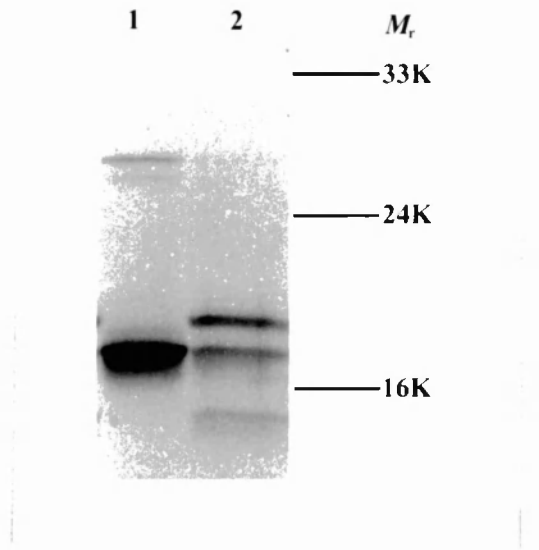
linked glycosylation acceptor site sequence, suggesting that eFGF may be glycosylated *in vivo* . *In vitro* translation of synthetic *eFGF* mRNA in the presence of canine pancreatic microsomes demonstrates that these predictions are likely to be correct and eFGF enters the secretory pathway by translocation into the lumen of the endoplasmic reticulum (ER). Fig. 1 shows the results of translating eFGF in rabbit reticulocyte lysate in absence or presence of microsomes. Lane 1 shows the primary translation product of eFGF which corresponds to the predicted molecular weight of 21000. The inclusion of microsomes leads to 2 more bands appearing about 2000 smaller and 2000 larger than the primary product. These probably represent processing products following the cleavage of the signal peptide and the sequential addition of N-linked glycosylation following translocation into the lumen of the microsome.

The specific activity of eFGF and bFGF proteins are similar. However, injection of *eFGF* mRNA is at least 100 times more effective than *bFGF* mRNA at causing autoinduction of animal caps (Table 1). It is presumed that this difference is due to the possession by eFGF of a signal for secretion, as it has previously been shown that the presence of a signal sequence substantially increases the inducing activity of FGF mRNAs (Thompson and Slack, 1992).

Mesoderm induction by eFGF

The specific activity of recombinant eFGF was determined by the serial dilution animal cap assay. Typical results are in the range of 10^5 to 10^6 units/mg of purified protein, which is very similar to that of bacterially produced *Xenopus* bFGF protein (Kimelman *et al.*, 1988; Green *et al.*, 1990). In common with other

Fig. 1. *In vitro* translation of eFGF.



Autoradiograph showing the products of translating synthetic *eFGF* mRNA in a rabbit reticulocyte lysate system. 5 µl of a 25 µl ^{35}S methionine labelled reaction was run on a 15% PAGE gel. **Lane 1** shows the product of translating *eFGF* mRNA in the absence of canine pancreatic microsomes. **Lane 2** shows the products of translating *eFGF* mRNA in the presence of canine pancreatic microsomes and shows the presence of additional bands from sequential signal cleavage and glycosylation.

Table 1 Autoinduction of mesoderm in animal caps injected with eFGF and bFGF mRNA

Injection	Uninduced	Induced	*n*
Water	14	0	14
bFGF mRNA			
5 pg	14	0	14
50 pg	11	0	11
500 pg	3	8	11
eFGF mRNA			
5 pg	0	12	12

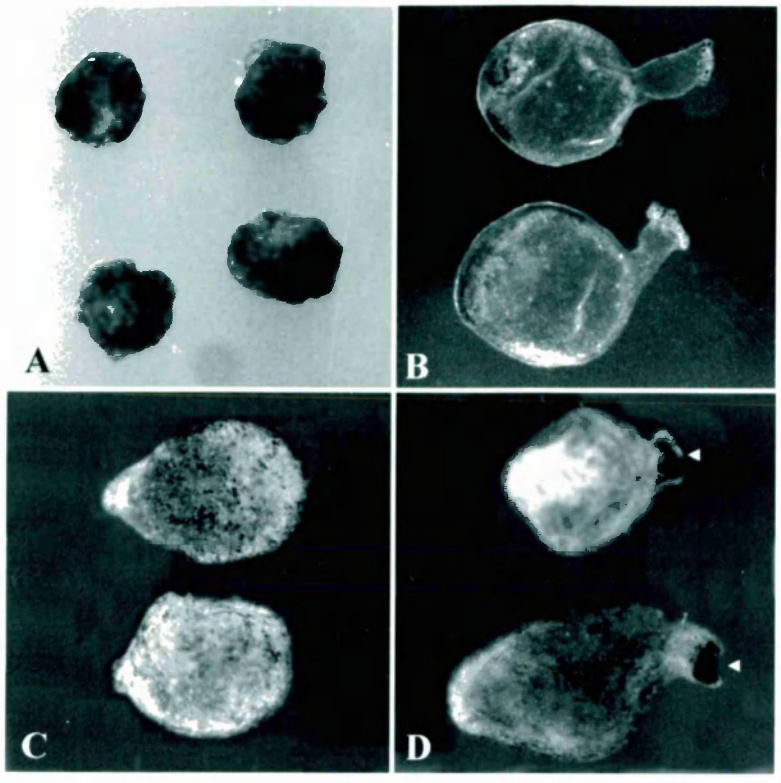
RNA was injected into both blastomeres of 2-cell stage embryos. Animal caps were removed at stage 9 and were cultured for 3 days. At this stage the presence of fluid-filled vesicles indicates the formation of mesoderm.

members of the FGF family the type of induction elicited by eFGF is ventrolateral in character. After 3 days of incubation the typical appearance of an eFGF induction is of a translucent fluid filled balloon-like vesicle. Fig. 2A shows uninduced control caps. Fig. 2B and C show inductions by bFGF and eFGF protein at about 10 units/ml. Histology of animal caps treated with eFGF shows that at low doses induction are extreme ventral in character, containing mesothelium and loose mesenchyme (Fig. 3B). At higher doses the inductions are more lateral in character containing increasing amounts of muscle (Fig. 3C). This has also been shown to be the case with increasing concentration of acidic FGF and basic FGF (Slack *et al.*, 1988).The formation of notochord is not detected in eFGF treated animal caps.

On a number of separate occasions the presence of darkened ring-like structures has been detected on the surface of eFGF induced vesicles (Fig. 2D). This has generally, but not always, been associated with higher doses of eFGF protein (up to 100 units/ml). Histology of these organised ectodermal structures bears similarities to the structure of the proctodeum (Fig. 3D, E and F). The proctodeum is an ectodermal derivative which has been shown to be enlarged and sometimes duplicated in embryos overexpressing eFGF after the midblastula transition (MBT) (Isaacs *et al.*, 1994; Pownall *et al.*, submitted).

The ventrolateral character of the mesoderm induced by eFGF has been confirmed with molecular markers. Fig. 4 shows that overexpression of *eFGF* mRNA in animal caps activates the expression of the early pan-mesodermal marker gene *Xbra*, and the ventrolateral mesoderm marker *Xwnt-8* but not the dorsal marker *noggin*.

Fig. 2. The appearance of animals cap explants following treatment with recombinant eFGF protein.



All animal caps were explanted at blastula stage 8 and cultured in either NAM/2 or NAM/2 + recombinant proteins for 3 days at 24^0 C until about control stage 40. **A** shows negative control uninduced animal caps cultured in NAM/2. **B** shows typical fluid filled vesicles formed following treatment with 10 units/ml of bFGF protein in NAM/2. **C** shows typical fluid filled vesicles formed following treatment with 10 units/ml of eFGF protein in NAM/2. **D** shows the appearance of vesicles formed following treatment with a high dose of eFGF protein (100 to 200 units/ml). White arrows indicate proctodeum-like structures which are frequently seen in animal caps treated with higher doses of eFGF protein.

Fig. 3. Histology of animals cap explants following treatment with recombinant eFGF protein.

Abbreviations: **epi**=epidermis. **me**=mesenchyme. **mst**=mesothelium. **mus**=muscle. **pcd**=proctodeum.

All animal caps were explanted at blastula stage 8 and cultured in either NAM/2 or NAM/2 + recombinant proteins for 3 days at 24^0 C until about control stage 40. **A** is a section from a negative control uninduced animal cap explant showing a mass of atypical epidermis. **B** is a section through an animal cap explant, treated with 10 units of eFGF protein, showing extreme ventral character of induction. **C** is a section through an animal cap explant, treated with 50 units/ml of eFGF protein, showing the presence of a large muscle block. **D** is a section through an animal cap explant, treated with 100 units/ml of eFGF protein, showing the typical appearance of the proctodeum-like ectodermal structures commonly found in animal caps treated with high doses of eFGF protein. The inset shows a section through the proctodeum of a stage 41 control embryo for comparison.

Fig. 3. Histology of animals cap explants following treatment with recombinant eFGF protein.

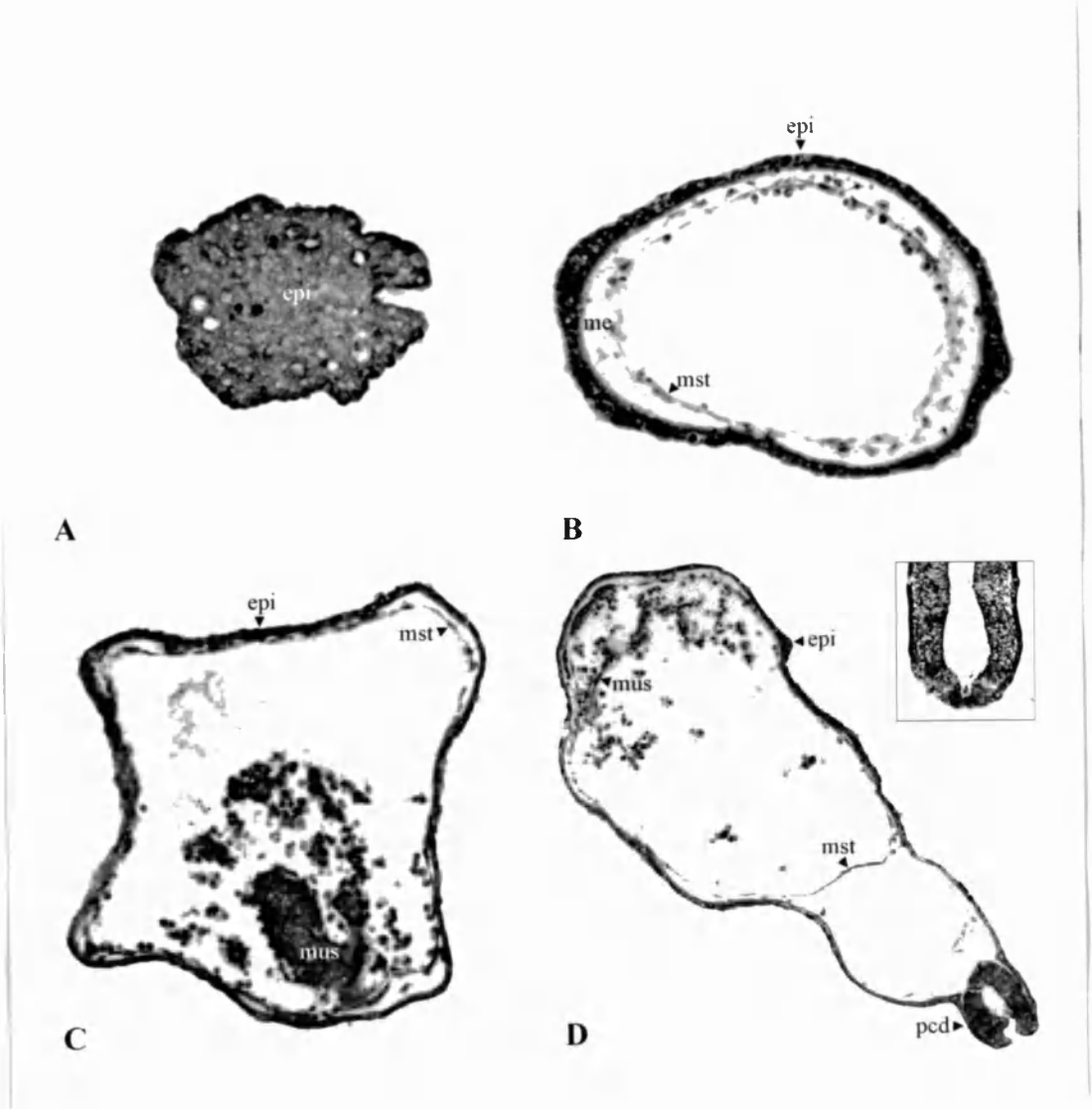
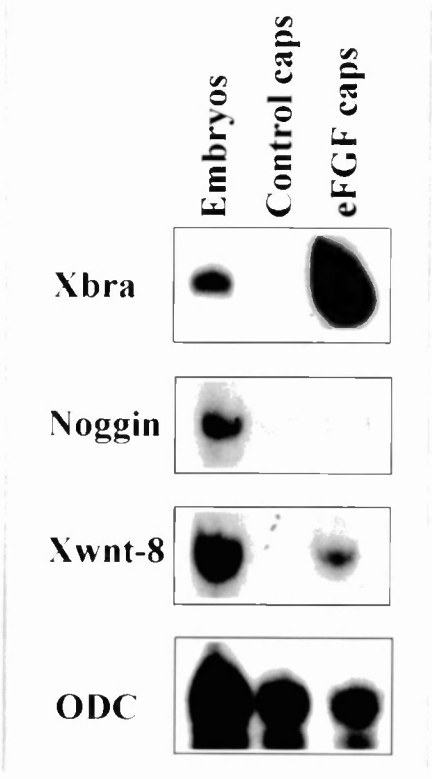


Fig. 4. RNAase protection analysis of mesodermal marker gene expression in animal cap explants taken from embryos injected with *eFGF* mRNA.

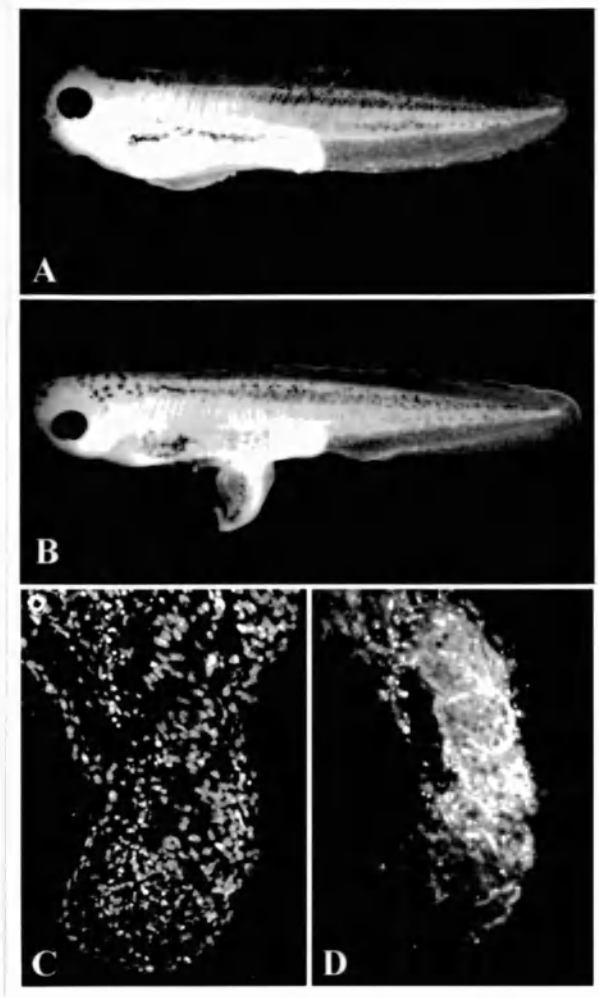


Animal cap explants were taken from embryos injected with 10 pg of *β-globin* control mRNA or 10 pg of *eFGF* mRNA and analysed by RNAase protection at stage 11. Hybridisation were carried out using 5 µg of total RNA for *Xbra* and 1 µg of total RNA for *noggin* and *Xwnt-8*. eFGF does not induce the expression of dorsal mesodermal markers such as *noggin*.

eFGF caps and the Einsteck-procedure

The Einsteck -procedure has an old pedigree in experimental embryology and has been used in the study of inductive interactions for many decades. More recently (Ruiz i Altaba and Melton, 1989b) showed that ventral-type implants such as bFGF-treated animal caps give rise to 'tail-like' protrusions containing segmented muscle blocks. The behaviour of fluorescently labelled eFGF treated caps has been investigated in the context of the 'Einsteck-procedure' and confirms that eFGF treated caps also behave as other a ventral character tissues in this assay. Fig. 5A and B show the resulting embryos following the Einsteck-grafting of an uninduced and eFGF treated animal cap. The posteriorally located tail-like protrusion is very similar to that produced by bFGF treated animal cap and ventral marginal zone (VMZ) Einsteck-grafts (Ruiz i Altaba and Melton, 1989b; Slack and Isaacs, 1994). Fig. 5C and D are sections through one of these protrusions and shows both graft (labelled) and host (unlabelled) tissues contribute to this structure. Note the presence of graft derived segmented muscle blocks in the protrusion (4/8 cases). Although muscle is commonly found in eFGF treated cap explants cultured in isolation it never has this segmented appearance. There is evidence that the segmented muscle in these protrusions requires a dorsalising influence from the host axis because UV ventralisation of the host eliminates segmented muscle in protrusions formed by Einsteck-grafts with VMZ implants (Slack and Isaacs, 1994).

Fig. 5. eFGF treated animal caps and the Einsteck-procedure



Stage 10 FDA labelled animal cap explants were implanted into the blastocoel of unlabelled host embryos and grown until stage 41. **A** shows a stage 41 control embryo that was implanted with an uninduced control animal cap. **B** shows a stage 41 embryo that results from implanting an animal cap that was treated with 20 units/ml of eFGF protein. Note the resulting tail-like protrusion. **C** is a fluorescence image of a section through the protrusion stained with DAPI to reveal cell nuclei. **D** is an image of a section through the protrusion. Tissue contribution from the graft is fluorescent. The protrusion contains both host and graft derived cells. Note the presence of a mass of graft derived segmented muscle.

Discussion

FGFs and secretion

Acidic FGF, basic FGF and FGF-9 lack a recognised secretory signal peptide. All other FGFs possess a classical hydrophobic amino terminal signal sequence and have been shown to enter the secretory pathway (Basilico and Moscatelli, 1992). The issue of secretion by the FGFs lacking a classical signal peptide is contentious. Mammalian FGF-9 appears to be secreted to some degree from COS cells and protein detected in the medium conditioned by these cells is glycosylated (Miyamoto *et al.*, 1993). This indicates that FGF-9 does in fact translocate to the endoplasmic reticulum (ER) and trans-golgi, suggesting the presence of a non-classical cryptic signal for secretion in its primary sequence. Furthermore, there is increasing evidence that acidic FGF, at least, can be released by certain cell-types by a novel mechanism, which does not involve the normal ER route for secretion (Jackson *et al.*, 1992).

Clearly the issue of secretion has to be taken into account when considering the role that the FGFs may play during early development. The data in this thesis strongly suggests that *Xenopus* eFGF is more efficiently secreted than *Xenopus* bFGF in early embryonic cells. Furthermore, *in vitro* translation indicates that eFGF enters the ER trans-golgi pathway undergoing sequential signal peptide cleavage and N-linked glycosylation. *Xenopus* FGF-3, which possesses a signal peptide, has been shown to be secreted from COS cells but this has not been tested in the embryo (Kiefer *et al.*, 1993a) The results for *Xenopus* FGF-9 are somewhat equivocal. Although FGF-9 enters the secretory

pathway, as judged by translocation into the pancreatic microsomes and core glycosylation, its mRNA has autoinducing activity intermediate between that of *bFGF* and *eFGF* (Song and Slack, 1996). This may indicate that although FGF-9 enters the secretory pathway it is not presented efficiently at the cell surface. A similar phenomenon has been shown for mammalian *FGF-3*, which is retained within the trans-golgi and is only slowly released at the cell surface (Kiefer *et al.*, 1993b).

eFGF and mesoderm induction.

In common with all other FGFs examined, the character of mesoderm induced by eFGF is of a ventrolateral character. Treatment with FGFs over a 100-fold range of concentrations shows that animal caps exhibit an extended dose response to these factors (Slack *et al.*, 1987; Slack *et al.*, 1988). At low doses of FGF, inductions are of an extreme ventral character. Such animal caps form vesicles consisting of an outer jacket of epidermis surrounding loosely packed mesenchyme and a layer of mesothelium. At higher doses the inductions are of a more lateral character with increasing quantities of muscle being found. However, even at the highest doses, explants taken from the animal pole region never form notochord in response to FGF treatment. Analysis of molecular markers indicates that in contrast to activin-like molecules, the FGFs are unable to induce the expression of *goosecoid*, *noggin* and *LIM* (Cho *et al.*, 1991; Smith and Harland, 1992; Taira *et al.*, 1992). These are genes expressed in the region of Spemann's organiser and considered to be markers of the most dorsal mesoderm. Furthermore in the Einsteck-procedure eFGF treated caps behave very much like other ventral character explants.

However, mesoderm induction by FGF can be modified to a more dorsal-type in number of ways. For example, it has been shown that the injection of *Xwnt*-8 mRNA into animal caps results in a more dorsal-type induction following FGF treatment (Christian *et al.*, 1992). This raises the possibility the FGFs may not only be involved in the formation of ventral-type but also dorsal-type mesoderm.

Of course the background to this whole discussion must be that the spatial expression data for the known *Xenopus* FGFs suggests that they are not likely to be part of the maternal vegetally localised mesoderm inducing signal (see Chapter 3). The low level of maternal FGF expression is predominantly in the animal hemisphere and, as will be discussed in later chapters, there is building evidence to support the view that this maternal FGF is a determinant of animal hemisphere competence to respond to the vegetal inducers.

The mesoderm inducing activity of *Xenopus* FGF-3 has not yet been tested but previously it has been shown that *in vitro* synthesised mammalian FGF-3 protein has weak mesoderm inducing activity in the animal cap assay (Paterno *et al.*, 1989). The receptor binding specificity of *Xenopus* and mammalian FGF-3 is very similar (Mathieu *et al.*, 1995), so it is likely that *Xenopus* FGF-3 is also active as a mesoderm inducing factor.

As has been discussed, zygotic expression of *eFGF* and *FGF-3* is activated in the marginal zone of the late blastula soon after the MBT. If the zygotic FGFs are mesoderm inducers *in vivo* they must be considered as secondary inducers to the primary inducers emitted by the vegetal hemisphere. Whether eFGF and FGF-3 accumulate to sufficient levels necessary to contribute to a secondary

phase of mesoderm induction in the late blastula before competence of the animal cells disappears is a moot point.

This chapter shows that eFGF has a potent activities in a number of *in vitro* assays. The following 2 chapters consist of an analysis of embryos in which the function of the FGF signalling pathway has been compromised and provides support for the view that FGFs are indeed required for the formation of both dorsal and ventral mesoderm during blastula stages. In keeping with the presented expression data it also indicates additional roles for the zygotically expressed FGFs in the gastrula and subsequent development.

Chapter 5

The phenotype of embryos overexpressing a dominant negative FGF receptor.

Introduction

The animal cap assay has provided much useful information on the properties of the FGFs as mesoderm inducing factors. However, definitive proof that the FGFs have an important role in early development must necessarily rely on experiments in which the action of the FGFs is inhibited *in vivo*. In developmental systems that are amenable to genetic manipulation, such as the mouse and *Drosophila*, this may be achieved by mutational and "gene knockout" procedures. Unfortunately this approach is not yet feasible in *Xenopus*. However, an increasing range of technologies are being developed which will allow the inhibition of a particular molecule or group of molecules.

One approach to inhibition, which has been used to great effect in *Xenopus*, is the construction of "dominant negative" forms of growth factor receptors. This approach relies on the fact that the receptors of many growth factors bind their ligands as dimers. Binding of ligand to the receptor complex leads to the activation of their intracellular kinase domains and cross phosphorlyation of the receptor components. In the case of the EGF, PDGF and FGF tyrosine kinase receptors, phosphorylation on specific tyrosine residues is a prerequisite for the activation of the downstream signal transduction pathway (Ullrich and Schlessinger, 1990; Egan and Weinberg, 1993; Johnson and Williams, 1993). Similar phosphorylation events are involved in signal transduction from members of the TGFß receptor family, but in this case the receptors have intracellular serine/threonine kinase activity. Dominant negative forms of the FGF, activin and BMP receptors have been constructed that lack their intracellular kinase domains. When synthetic mRNAs coding for these mutant

forms are injected into the early embryo they are translated, and the resulting mutant proteins are able to form unproductive dimers with the endogenous wild-type receptor. If the mutant receptors are present in excess it can be shown that, in the case of the FGF, activin and BMP mutant receptors, animal caps are refractory to induction by the respective ligands (Amaya *et al.*, 1991; Graff *et al.*, 1994; Hemmati-Brivanlou and Melton, 1992). The dominant negative receptor approach has the advantage that, due to the cross reactivity of ligands and their receptors, it is likely that a given inhibitory mutant will block the activity of a whole group of ligands and possibly closely related receptors, as has been shown to be the case for a dominant negative form of the FGF-R1 (Ueno *et al.*, 1992). As such, it will serve to highlight those processes which require the activity of that group of signalling molecules. Of course the downside of this is that the use of a dominant negative receptor will not necessarily give any information as to the identity of the specific ligands that are important. In this way, it can be seen that the complete absence of mesoderm in embryos overexpressing the dominant negative activin receptor indictes a crucial role for an activin-like molecule in mesoderm induction, however the identity of this molecule is at present unclear (Hemmati-Brivanlou and Melton, 1992; SchulteMerker *et al.*, 1994b).

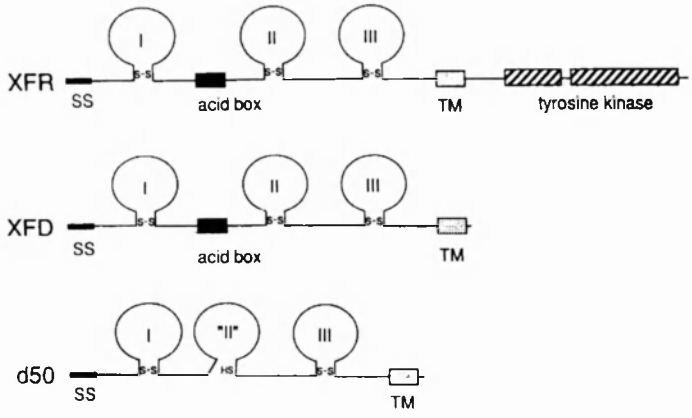
The signal transduction pathway used by tyrosine kinase growth factor receptors such as the FGF-receptor is now very well characterised (reviewed by Egan and Weinberg, 1993). The treatment of cells with FGF leads to a number of alterations in the intracellular environment including an increase pH, Ca^{++} levels, turnover of phosphoinositides and increase in the phosphorylation of a number of proteins (reviewed by Johnson and Williams, 1993). Overexpression of

constitutively active and inhibitory forms of key downstream components of this pathway have helped define those components which are not only necessary but sufficient for FGF mediated mesoderm induction. Experiments of this type have shown that the ras, raf, MEK, MAP kinase pathway is of paramount importance in mesoderm induction by the FGFs. However, the polyphosphoinositide metabolic pathway appears to be less important. Phospholipase c-gamma, which is a key enzyme involved in inositol phosphate metabolism, has been shown to associate with the FGF receptor in response to FGF treatment in a number of cell types including animal caps cells (Ryan and Gillespie, 1994). However, this association or the activity of phosphatidylinositol 3-kinase is not required for FGF mediated mesoderm induction (Muslin *et al.*, 1994b; Umbhauer *et al.*, 1995).

The dominant negative FGF-receptor used in this study is that of (Amaya *et al.*, 1991). They constructed several deletion mutant form of the Xenopus FGF receptor 1 (Fig. 1) and showed that one of them (XFD), which lacks its intracellular tyrosine kinase domain, is capable of acting as a dominant inhibitor of FGF activity. Injection of RNA coding for this mutant receptor blocks FGF signalling in a number of assays including the animal cap assay and a calcium ion release assay in oocytes. Furthermore, the specificity of this effect was demonstrated by the rescue of receptor function by the coinjection of an excess of wild-type receptor RNA. A control mutant (d50), with an additional 50 amino acid deletion of a region between IgG domain 1 and 2 containing the so-called 'acid box', does not block FGF signalling (Fig. 1). One important issue with any overexpression study using injected synthetic mRNA is the stability of the message and protein translated from it. Protein produced from injected XFD mRNA (Amaya *et al.*,

1993) and the mRNA of a myc epitope tagged dominant negative FGF receptor (Pownall, personal communication) persists until at least the end of the neurula stage. This means that this reagent is an effective way of blocking FGF signalling through blastula, gastrula and neurula stages.

Fig. 1 *Xenopus* FGF receptor constructs



Schematic diagram showing the structure of the wild-type FGF receptor 1 (XFR), the dominant negative FGF receptor (XFD), lacking an intracellular tyrosine kinase domain, and the control non-functional receptor (d50), lacking both the acid box and intracellular tyrosine kinase domain.

Extensive use of the XFD dominant negative mutant form of the FGF receptor has been made in this thesis and elsewhere (Amaya *et al.*, 1993; Cornell and Kimelman, 1994; Cornell *et al.*, 1995; Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). However, the description of the phenotype of XFD mRNA injected embryos in the original study by (Amaya *et al.*, 1991) was not very detailed. In order to gain a better understanding of the requirement for FGF signalling during early development it was decided to undertake a detailed study of the morphology and histology of XFD injected embryos. This chapter contains the results of this study.

Injection of XFD mRNA into the early embryo leads to a characteristic phenotype in which anterior structures are relatively unaffected but in which there is a complete loss of trunk and tail development. Dorsal and ventral injections of XFD show that much of the XFD phenotype can be accounted for by a sensitive requirement for FGF signalling on the dorsal side of the embryo. Inhibition of FGF signalling on the dorsal side results in the failure of the dorsal mesoderm to involute and extend along the developing anteroposterior axis. Instead, dorsal tissues spread laterally and ventrally around the blastopore resulting in its failure to close during gastrulation.

Histology of XFD injected embryos and gastrula stage explants from XFD injected embryos shows that inhibition of FGF signalling results in a great reduction in the amount of the dorsal mesodermal tissue types notochord and muscle. More ventral tissue types, such as kidney, mesenchyme and mesothelium, are also similarly affected. Interestingly, the amount of the most ventral mesodermal tissue type blood is not reduced, and is in fact somewhat increased in lateral explants from XFD embryos. There is some reduction of the amount of neural tissue in XFD injected embryos but it is never completely absent. Even in the most severely affected embryos anterior neural tissue is present, although its pattern is deranged. Einsteck and grafting experiments with dorsal marginal zone explants from XFD embryos suggest that the activity of the Spemann organiser is compromised by the inhibition of FGF signalling.

Materials and Methods

Embryo injections and manipulations

The dominant negative FGF receptor (XFD) and control receptor (d50) constructs are those used by Amaya *et al.*, (1991). The pSP64-eFGF plasmid is as described by Isaacs *et al.*, (1994). Embryos for animal caps were injected into both blastomeres in the animal pole region following first cleavage. Animal caps were taken at stage 9 as per general methods and scored for vesicle formation after 3 days of culture. All XFD and d50 injections for phenotype and tissue differentiation experiments were targeted to the pigment boundary of blastomeres in the 4-cell stage embryo. Embryos were cultured until control stage 40-41 for scoring by phenotype and histology.

Gastrula stage explants were made by dissecting stage 10.5 embryos in NAM into dorsal, lateral and ventral quadrants using a microsurgical knife. Explants were allowed to heal and then cultured in NAM/2 until control stage 41. For the Einsteck-procedures and organiser graft, embryos were injected into all 4 blastomeres at the 4 cell stage with 1 ng of XFD mRNA in 5 nl of DEPC treated water. Einsteck-grafts were 60^0 wedges of tissue from either the dorsal or ventral marginal zone of stage 10.5 embryos. Explants extended from the blastopore lip to the floor of the blastocoel and care was taken to remove as much vegetal core material as possible. The explants were grafted into the blastocoel of unlabelled hosts as per general methods. Organiser grafts were carried out by implanting dorsal explants from XFD and control embryos into a slit cut into the ventral marginal zone of hosts at stage 10.5. Embryos were allowed to heal in NAM/2 and then cultured until control stage 40 in NAM/4.

Results

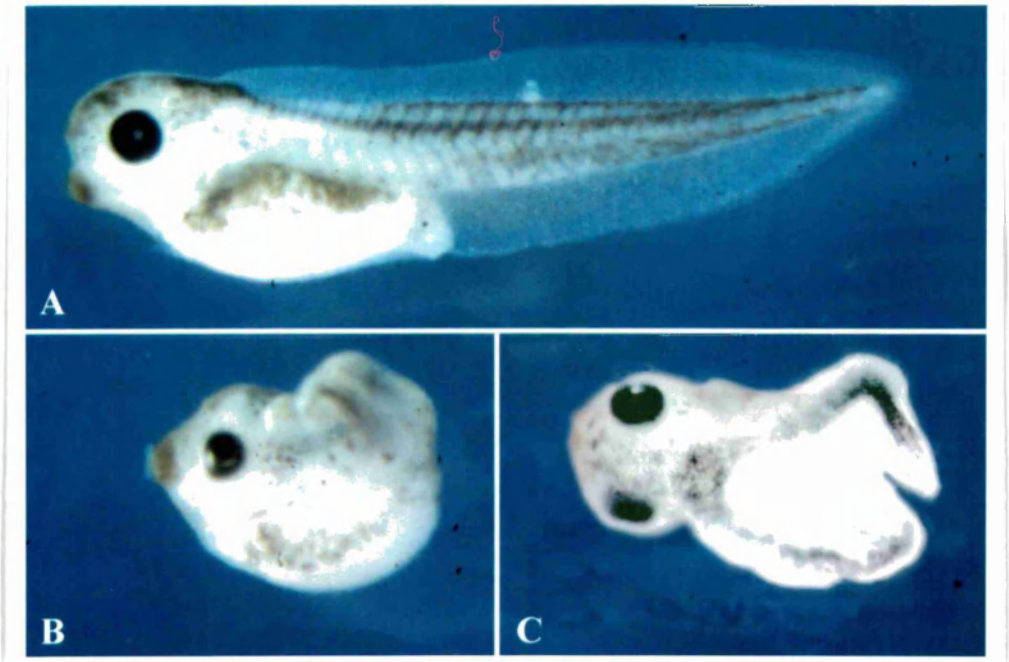
The phenotype of the dominant FGF receptor injected embryo

Fig. 2A, B and C show the typical appearance, after 3 days of development, of control embryos and embryos that have been injected with 1 ng of dominant negative FGF receptor (XFD) mRNA into each blastomere at the 4-cell stage. Compared to controls there is a massive reduction in the development of trunk and tail structures. A striking feature of these embryos is that the blastopore fails to close. Anterior development is relatively unaffected and cement glands and eyes are generally present in these embryos. The originally mooted function of the FGFs in early *Xenopus* development was as ventral mesoderm inducing agents. However, as has already been discussed in Chapter 2 the expression of the known *Xenopus* FGFs is not restricted to the ventral side of the embryo. Furthermore, the pronounced reductions in axial structures in XFD injected embryos strongly suggests that the FGFs must be intimately involved in dorsal development. A number of experiments have been performed in order to understand better the requirement for FGF signalling on the dorsal and ventral side of the embryo.

Morphology of embryos resulting from dorsal and ventral injections of XFD

The effects on gross embryo morphology resulting from high (1 ng) and low (0.1 ng) dose injections of XFD mRNA into both blastomeres on the dorsal or ventral side of embryos at the 4-cell stage was examined at different developmental stages.

Fig. 2. The phenotype of *Xenopus* embryos injected with mRNA coding for a dominant negative FGF receptor (XFD).



Embryos were injected with 1 ng of XFD mRNA or control d50 mRNA into each blastomere of the 4-cell stage embryo and cultured until the swimming larva stage 41. **A** is side view of a d50 mRNA injected control embryo (anterior to the left, dorsal to the top). **B** is a side view of an XFD mRNA injected embryo. Note the quite normal head and vestigial tail and trunk structures. **C** is a view from above down on to the open blastopore of an XFD mRNA injected embryo. Note the exposed yolk mass and formation of head close to the open blastopore.

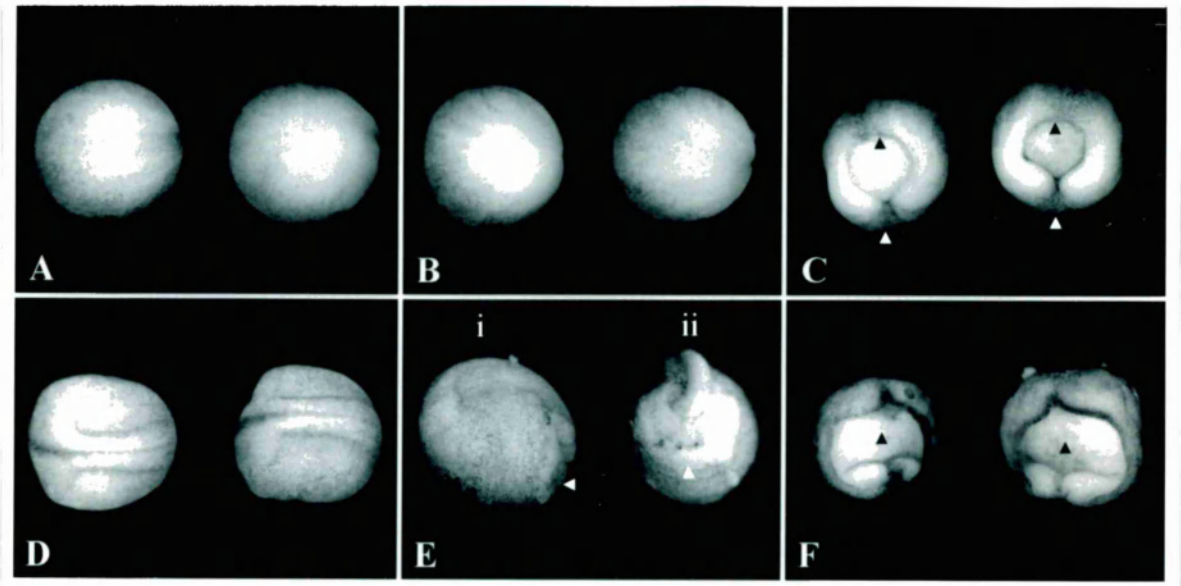
The gastrula stage

The initial stages of gastrulation in control and XFD embryos are very similar. The formation of the dorsal blastopore lip is not delayed in XFD embryos. However, as gastrulation proceeds it is apparent that the closure of the blastopore is inhibited in embryos that have been injected with even a low dose (2x 100 pg) of XFD mRNA on the dorsal side. A dorsal view of control uninjected embryos at late gastrula stage 13 shows the outlines of the forming neural plate and closed blastopore at the posterior of the embryo (Fig. 3A). The appearance of embryos injected with a low dose of XFD on the ventral side is identical to that of control embryos (Fig. 3B). In contrast to ventrally injected embryos, Fig. 3C is a vegetal view of low dose dorsal injected XFD embryos at stage 13. Note the open blastopore and exposed yolk mass. There is a characteristic 'doughnut-like' thickening of tissue around the margin of the blastopore. This thickening appears to result from a net movement of tissue around the lateral and ventral margins of the blastopore from the dorsal lip region. The ventral limit of the extension around the blastopore is marked by a furrow or constriction close to the ventral midline of the blastopore. These data indicate that the open blastopore aspect of the XFD phenotype arises from a sensitive effect on FGF signalling on the dorsal side of the embryo.

The neurula stage

Fig. 3D shows the appearance of control embryos at the end of the neurula stage 20 following the fusion of the neural folds. As is the case at the end of the gastrula stage, embryos that have been injected with a low dose of XFD mRNA on the

Fig. 3. The development of the dominant negative FGF receptor phenotype through gastrula and neurula stages.



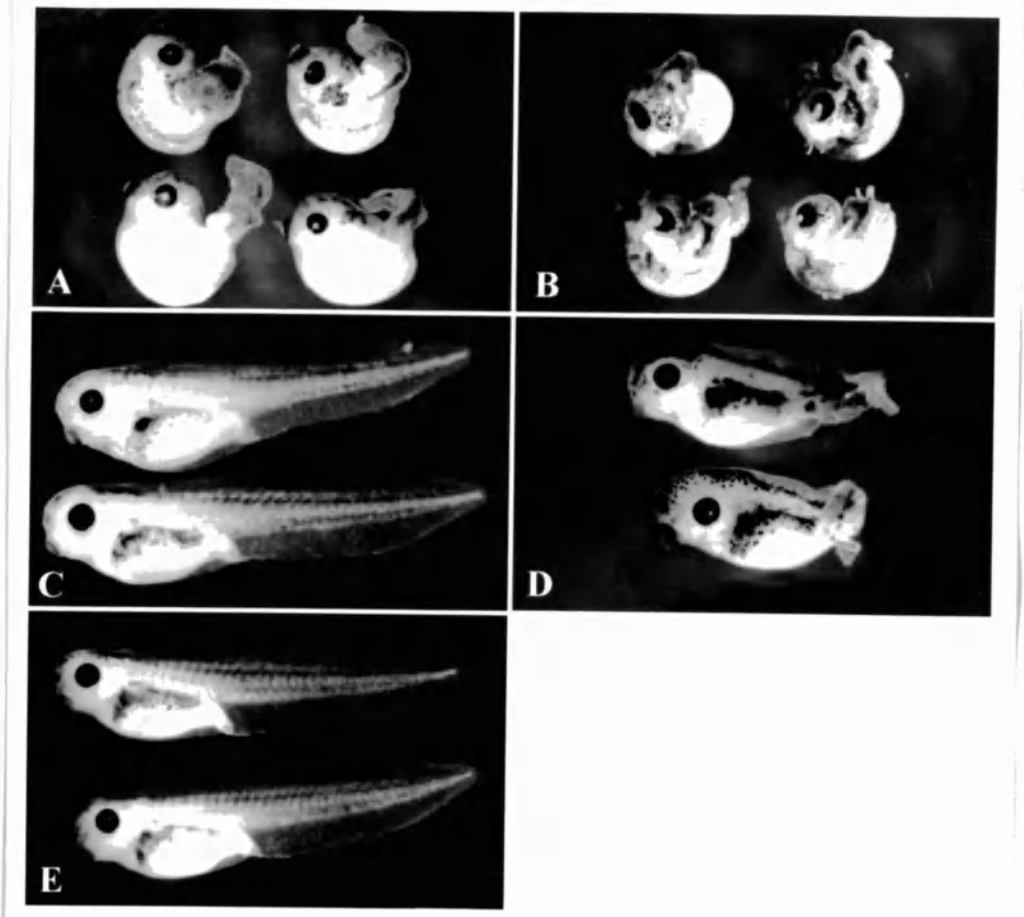
Embryos were injected with low (100 pg) or high (1 ng) doses of XFD mRNA into either both ventral or dorsal blastomeres at the 4-cell stage and cultured to the relevant stages. **A** is a dorsal view of uninjected control embryos at late gastrula/early neurula stage 13 embryos (anterior to the left). **B** is a dorsal view of stage 13 embryos injected into the ventral side with a low dose of XFD mRNA. **C** is a vegetal view of stage 13 embryos injected into the dorsal side with a low dose of XFD mRNA showing the open blastopore. Black arrows indicate the dorsal lip. White arrows indicate the constriction furrow on the ventral side of the blastopore. **D** is a dorsal view of control uninjected late neurula stage 20 embryos. **E** are stage 20 embryos injected with a high dose of XFD mRNA into the ventral side. (i) is a side view (anterior to the left, dorsal to the top). (ii) is a posterior view. Note normal axial development and the gaping appearance of the ventral blastopore lip (white arrows). **F** is vegetal view of stage 20 embryos injected into the dorsal side with a high dose of XFD mRNA showing the pronounced open blastopore and exposed vegetal yolk mass (black arrows) (dorsal to the top).

ventral side are quite normal (data not shown). Although the developing axis of embryos that have received a high dose (2x 1 ng) injection of XFD mRNA on the ventral side appears quite normal, a close examination of the blastopore of these embryos (Fig. 3E) reveals that the ventral lip has failed to close and has a somewhat gaping appearance. In embryos injected with XFD mRNA on the dorsal side the open blastopore is even more pronounced than at the end of gastrulation (Fig. 3F). These data suggest that the failure of the blastopore to close results in XFD embryos mainly results from a sensitive effect on FGF signalling on the dorsal side of the embryo. However, the gaping appearance of the ventral blastopore lip in high dose ventrally injected embryos also suggest a role for FGF signalling during ventral development.

The swimming larva stage

The type of effects obtained from dorsal versus ventral injections of XFD and the resulting phenotypes at swimming larva stage 40 were classified according to the following criteria. A class 3 embryo is defined as having a completely open blastopore. The head forms very close to the dorsal blastopore lip and is relatively normal in appearance but there is a tendency towards cyclopia and even loss of eyes in the more extreme examples. The trunk and posterior axis is split and very much reduced in length and volume, forming two characteristic small "wings" of tissue along the margins of the lateral blastopore lip. This phenotype is characteristic of both low and high dose XFD injections (Fig. 4A and B). A class 2 embryo shows varying degrees of splitting of the trunk and tail, and consequent reductions in these structures, caused by incomplete closure of the blastopore.

Fig 4. The phenotypes of swimming larva stage embryos resulting from dorsal and ventral injections of XFD mRNA.



Embryos were injected with low (100 pg) or high (1 ng) doses of XFD mRNA into either both ventral or dorsal blastomeres at the 4-cell stage and cultured to the swimming larva stage 41. **A** and **B** are typical class 3 embryos resulting from the dorsal injection of low (**A**) and high (**B**) doses of XFD mRNA. **C** are quite normal class 0 embryos resulting from the low dose ventral injection of XFD mRNA. **D** are typical class 1 embryos resulting from the high dose ventral injection of XFD mRNA. **E** are quite normal class 0 embryos resulting from the high dose injection of d50 control mRNA into all blastomeres of the 4-cell stage.

In a class 1 embryo, which is typical of high dose ventral injections of XFD, there is complete closure of the blastopore dorsally and laterally. However, ventrally the blastopore lip of the class 1 embryo has a slightly gaping appearance, especially at earlier stages. In class 1 embryos there are varying degrees of reduction in the number and volume of trunk and tail somites (Fig. 4D). The remaining axis tends to be bent ventrally. On this scale class 0 represent embryos with normal appearance as is typical with low dose ventral injections of XFD and control (d50) injected embryos (Fig. 4C and 4E).

Table 1 further illustrates the dramatic difference in the phenotype produced by dorsal versus ventral injections. In addition, as judged by effects on morphology, the dorsal side of the embryo is much more sensitive to FGF inhibition than is the ventral side. Even low doses of XFD mRNA injected into dorsal blastomeres give rise to a high proportion of class 3 embryos. The same dose injected ventrally has no obvious effect on the embryos. However, high dose ventral injections give rise to a high proportion of class 1 embryos.

Table 1 Phenotype produced by dorsal and ventral injections of dominant negative FGF receptor (XFD) mRNA.

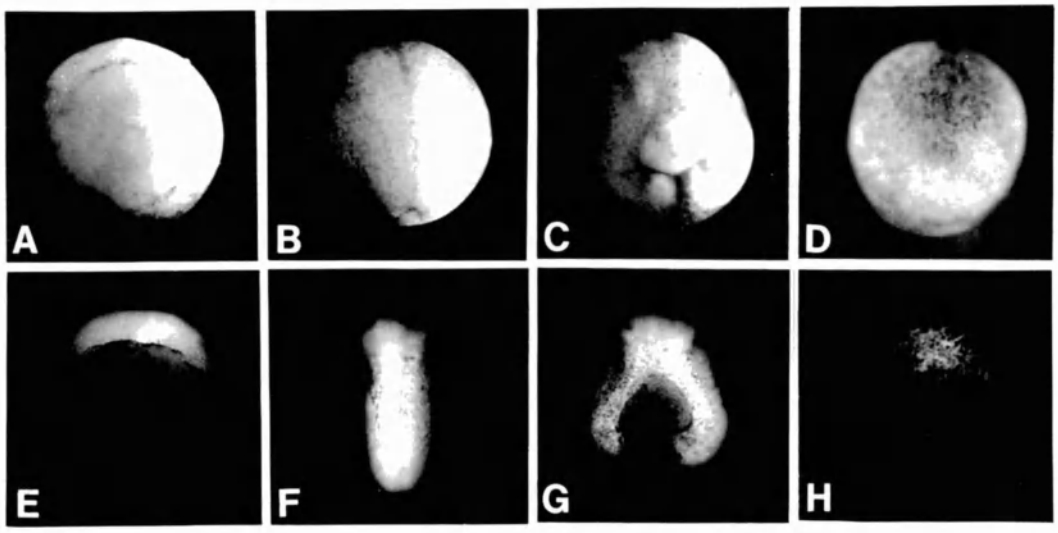
Injection	Class 0	Class 1	Class 2	Class 3	Other	*n*
None	43	3	0	0	2x double axis	48
2x 1 ng d50 mRNA						
Dorsal	11	1	0	1	1x μcephalic	14
Ventral	15	1	0	0	0	16
2x 0.1 ng XFD mRNA						
Dorsal	6	2	0	13	0	21
Ventral	14	1	0	0	1x μcephalic	16
2x 1 ng XFD mRNA						
Dorsal	0	0	0	23	0	23
Ventral	0	13	6	0	1x runt	20
Dorsal+Ventral	0	0	0	11	0	11

XFD is the dominant negative FGF receptor and d50 is a control non-functional mutant receptor. Dorsal blastomeres were injected with the relevant mRNAs at the 4-cell stage. They were scored at stage 40 according to the criteria described in the text.

Cell movements in XFD injected embryos

The first abnormality that is apparent in embryos injected with the dominant negative FGF receptor on the dorsal side is the failure of the blastopore to close at the end of gastrulation. From this it is clear that inhibition of FGF signalling must affect cell movements on the dorsal side of the embryo during gastrulation. The nature of this effect on the behaviour of cells on the dorsal side of XFD embryos has been examined by coinjecting a fluorescent dextran lineage label and XFD mRNA into both dorsal blastomeres at the 4-cell stage. This protocol enables the cell movements on the dorsal side of the embryo to be followed *in vivo* during gastrulation. Fig. 5E shows a whole-mount fluorescent microscope vegetal view of such an embryo at early gastrula stage 10 with only the dorsal half labelled. The incident light image shown in Fig. 5B shows complete closure of the blastopore in a control embryo at late gastrula (stage 13). Figure 5F shows the pattern of labelling in such a control embryo in which the dorsal mesoderm has involuted and extended along the forming antero-posterior axis. In contrast, Figure 5C and G show that, in embryos injected dorsally with XFD mRNA the blastopore fails to close and the dorsal mesoderm does not extend to form a normal axis but instead spreads laterally around the blastopore and pushes as two horns of axial tissue onto the ventral side of the embryo. Therefore it would seem likely that in such embryos the axial mesoderm instead of extending along the anteroposterior axis, extends and drives around the margin of the blastopore, resulting in the failure of blastopore closure seen in class 3 embryos. The animal view of an XFD injected embryo in Fig. 5D and H shows that there are some labelled cells on the dorsal side of the animal hemisphere but there is clearly no

Fig. 5. Cell movements in embryos injected with dominant negative FGF receptor (XFD) mRNA.



(**A** and **E**) show incident and fluorescent light images of the vegetal view of an early gastrula embryo (stage 10) that was coinjected with 250 pg of d50 control mRNA and 10 nl of FDA lineage label (12.5 mg/ml) into the dorsal blastomeres at the 4-cell stage. Note the fluorescent marker is confined to region of the dorsal lip.

(**B** and **F**) show the dorsal view of similarly injected a late gastrula stage 13 control embryo. Note that in (**B**) the blastopore is closed and that in (**F**) the labelled dorsal mesoderm has extended along the antero-posterior axis.

(**C** and **G**) show a vegetal view of a late gastrula stage 13 embryo that was coinjected with 250 pg XFD mRNA and 10 nl of FDA lineage label (12.5 mg/ml) into the dorsal blastomeres at the 4-cell stage. Note that in (**C**) the blastopore has failed to close leaving the yolk mass exposed and that in (**D**) the labelled mesoderm has split and extended laterally around the blastopore towards the ventral side of the embryo.

(**D** and **H**) show an animal view of the same embryo in (**C** and **G**). Note that there is no axial extension towards the animal pole region but there is some migration of anterior mesendodermal cells on the dorsal side of embryo (top).

axial extension towards the animal pole region. It is likely that this dorsal labelling is due to the involution of anterior mesendodermal cells which do not undergo convergent extension but rather exhibit active crawling behaviour. These cells contribute to the well formed anterior structures of FGF dominant negative embryos and do not appear to be as sensitive to the effects of FGF inhibition as the axial mesoderm.

In normal development the extension of the dorsal mesoderm along the developing anteroposterior axis is driven by mediolateral cellular intercalation that leads to a narrowing of the cell array at the dorsal midline (Keller *et al.*, 1992a). Cells in the neural plate also exhibit similar behaviour, which accounts for the elongation and narrowing of this structure during development (Keller *et al.*, 1992b). In XFD injected embryos the dorsal mesodermal cells and perhaps dorsal ectoderm cells still undergo extension movements but instead of the dorsal cell array narrowing at the dorsal midline the cellular array would appear to narrow along the animal vegetal axis driving extension movements around the open blastopore. A simplistic view of this phenomenon might suggest that the cells of the dorsal axis are still undergoing cellular intercalation resulting in a narrowing and extension of the cell array but inhibition of FGF signalling results in this occurring in the wrong direction.

Histology of XFD injected embryos

In order to gain a better idea of the effects which lead to the formation of the typical dorsal and ventral phenotypes, histology was carried out on embryos that

have been injected with high doses (1 ng) of XFD mRNA either into all 4 blastomeres at the 4-cell stage or just the ventral or dorsal blastomeres.

Histology of control embryos

As a comparison for the XFD injected embryos transverse sections from stage 40 control embryos are shown. Fig. 6A shows a section through the head at the level of the eyes. Fig. 6B shows a section through anterior trunk region. Fig 6C shows a section through the mid trunk.

Histology of embryos injected into both dorsal and ventral blastomeres

Embryos were injected into all blastomeres at the 4-cell stage and cultured until control stage 40. The phenotype of these embryos is generally similar to the class 3 embryo described above but in extreme cases, in addition to loss of trunk and tail structures, there can be loss of anterior structure such as eyes. Fig. 6D is a section through the anterior of an extreme case following injection of 1 ng of XFD mRNA into all 4 blastomeres and shows the complete absence of axial structures such as somites and notochord. In this particular case the eyes were absent but note the presence of a large block of disorganised neural tissue. In these embryos there is obviously a large reduction in the amount of mesodermal differentiation. This includes most mesodermal tissue-types such as notochord, muscle, kidney and mesenchyme. Blood, however, seems to be less severely affected and a sizeable blood island can be seen in Fig. 6D. Fig. 6E is a section through the region of the open blastopore from the same embryo (the exposed yolk mass is to the left), note the absence of any differentiated mesodermal tissue types. The presence of large amounts of wrinkled atypically thickened epidermis

Volume 3

The Fibroblast Growth Factor Family in the Early Development of *Xenopus laevis*.

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A thesis submitted to the Life Sciences Department of the Open University for the degree of PhD, May 1996.

Imperial Cancer Research Fund,
Developmental Biology Unit,
Department of Zoology,
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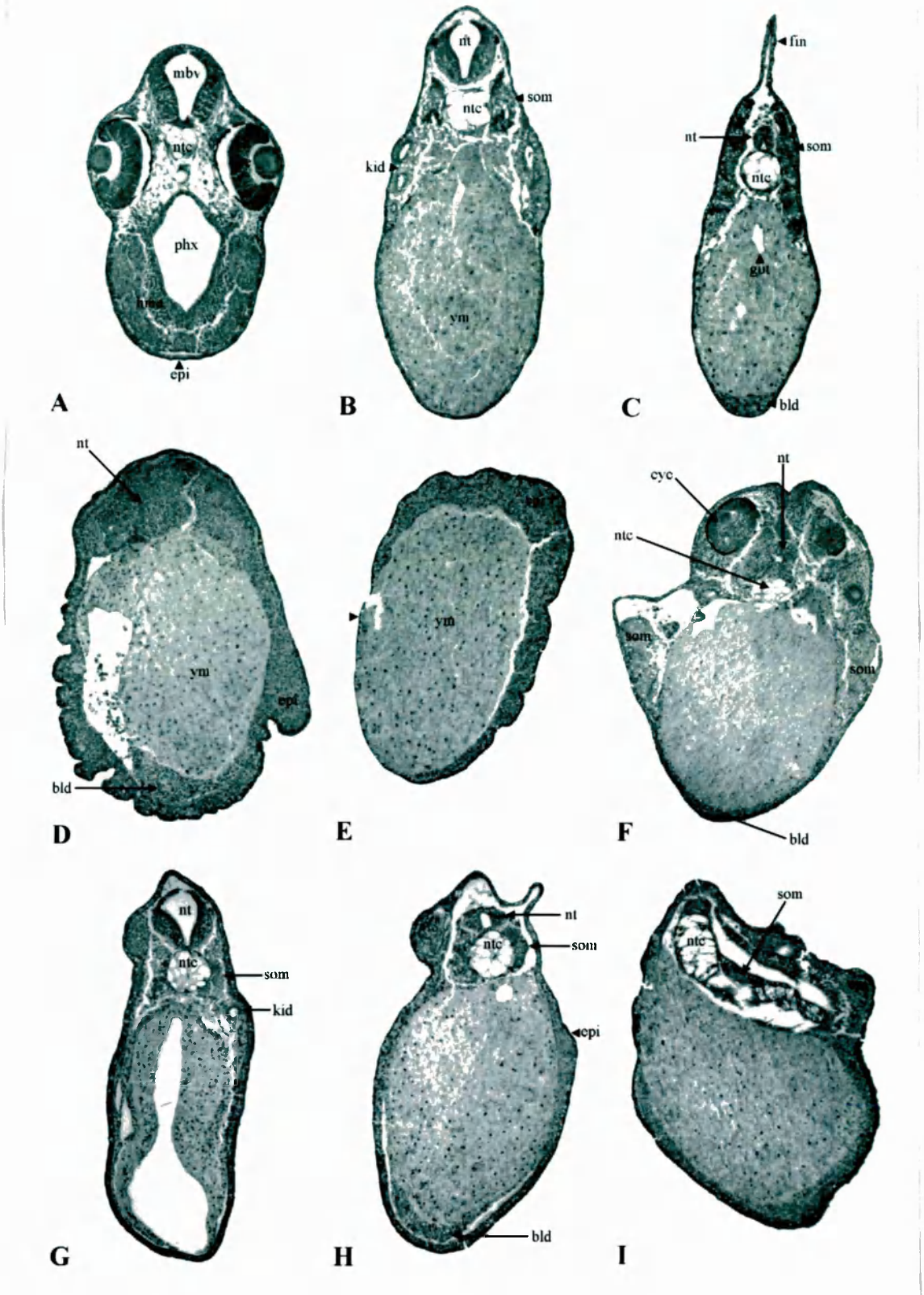
Fig. 6. Histology of embryos injected with dominant negative FGF receptor (XFD) mRNA.

Abbreviations: **bld**=blood. **epi**=epidermis. **hme**=head mesenchyme. **kid**=pronephric kidney tubule. **mbv**=midbrain ventricle. **nt**=neural tube. **ntc**=notochord. **phx**=pharyngeal cavity. **som**=somitic muscle. **ym**=yolk mass

Embryos were injected with 1 ng of XFD mRNA into either all 4 blastomeres or both ventral blastomeres or both dorsal blastomeres at the 4-cell stage and cultured until stage 41. **A** is a transverse section through the head of an uninjected control embryo at the level of the eyes. **B** is transverse section through the anterior trunk region of an uninjected control embryo. **C** is a transverse section through the mid trunk region of an uninjected control embryo. **D** is a transverse section through the anterior of an extreme XFD phenotype embryo produced by injecting all 4 blastomeres at the 4-cell stage with XFD mRNA. **E** is a section through the region of the open blastopore of the same embryo. The exposed vegetal yolk mass is to the left (black arrow). **F** is a transverse section through the head of a typical class 3 embryo resulting from the dorsal injection of XFD mRNA. **G**, **H** and **I** are transverse sections through class 1 embryos resulting from the ventral injection of XFD mRNA. **G** is at the level of the anterior trunk. **H** is at the level of the mid trunk. **I** is at the level of the posterior trunk.

Fig. 6. Histology of embryos injected with dominant negative FGF receptor (XFD) mRNA.



in these embryos suggests a failure in mesoderm induction resulting in abnormal production of ectodermal derivatives.

Histology of XFD dorsal injected embryos

Embryos were injected into both dorsal blastomeres at the 4-cell stage and cultured until control stage 40. The phenotype of these embryos is typical of the class 3 embryo described previously. Fig. 6F shows a section of through the head of a typical class 3 embryo just anterior to the region where the vestigial axis splits around the open blastopore. A small amount of notochord can be seen in this section but it is frequently absent in such embryos. Unlike embryos receiving 4 cell injections some somitic muscle is usually present, although the total amount is very much reduced. In this section it can also be seen that the pattern of the somites is disturbed. Unlike in control embryos the somites do not abut the notochord in the mid line but are split and are found in a more lateral position. Also note the presence of a well formed eye and large amounts of neural tissue. Although the external morphology of the 4 cell injected embryos and the dorsally injected embryos is generally similar, histology reveals that effect on mesodermal tissue differentiation is more severe in the 4 cell injected embryos.

Histology of XFD ventral injected embryos

Embryos were injected into both ventral blastomeres at the 4-cell stage and cultured until control stage 40. As might be expected from external morphology the effects on tissue differentiation and pattern are much less severe in ventrally injected embryos. Fig. 6G is section through a typical class 1 embryo at the level

of the anterior trunk and shows a somewhat normal appearance apart from a unilateral reduction in the amount of pronephric tubules. In mid trunk regions (Fig. 6H), although the amount of notochord and neural tissue is quite normal there is a large reduction in the amount of somite. Again differentiation of blood, which is the most ventral tissue-type, is not greatly affected and well defined blood islands can be seen in Fig. 6H. The reduction in somite development is even more dramatic in the posterior trunk region, where, even in the presence of large amounts of notochord, somitic muscle is almost completely absent (Fig. 6I). These data again highlight that the differentiation of somitic muscle is very sensitive to FGF inhibition. In the case of ventrally injected embryos the effect on the differentiation trunk somites is very much in keeping with fate map projections, which show that much of the prospective muscle forming region is derived from the ventral half of the embryo (Dale and Slack, 1987a). Just as with the 4-cell and dorsally injected embryos there appears to an abnormally thickened epidermis, particularly in posterior regions, which is suggestive of there being a failure in mesoderm induction in ventral regions and a shift towards the differentiation of more ectodermal derivatives.

The specification of dorsal, lateral and ventral explants from early gastrula XFD injected embryos

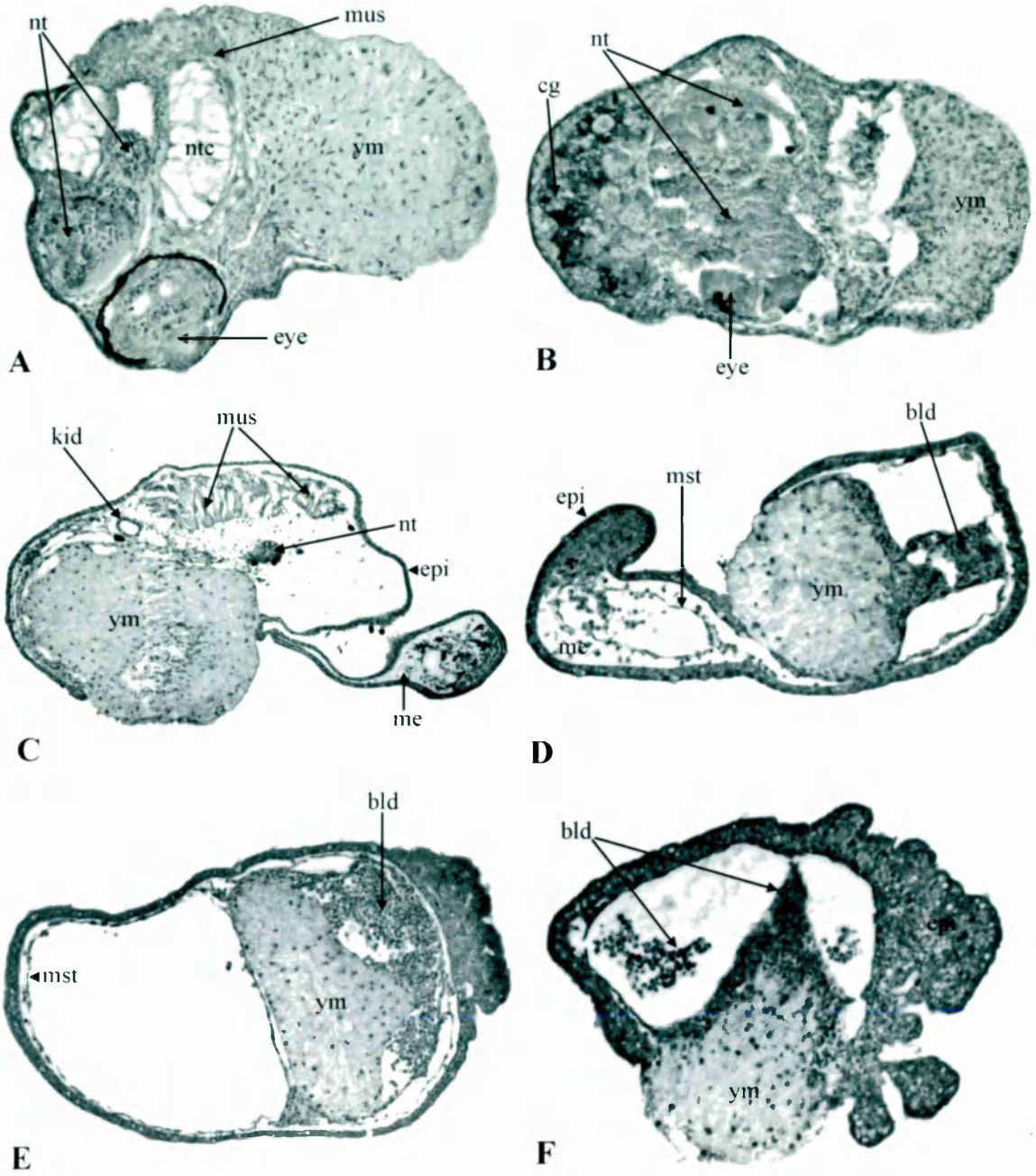
The data above would seem to indicate a role for FGF signalling in the differentiation and patterning of the mesoderm in *Xenopus*. However, the aberrant cell movements in XFD embryos must result in the abnormal juxtapositioning of tissues during gastrulation. It is known that during normal

Fig. 7. Histology of dorsal, lateral and ventral explants taken from gastrula stage embryos injected with dominant negative FGF receptor (XFD) mRNA.

Abbreviations: **bld**=blood. **cg**=cement gland. **epi**=epidermis. **kid**=pronephric kidney tubule. **me**=mesenchyme. **mst**=mesothelium. mus=muscle. **nt**=neural tissue. **ntc**=notochord. **ym**=yolk mass.

Dorsal, lateral and ventral embryo quarters were explanted at gastrula stage 10.5 from uninjected controls and embryos injected with 1 ng of XFD mRNA into each blastomere at the 4-cell stage. Explants were cultured in isolation until control stage 41. **A** shows a section through a control dorsal quadrant explant. **B** shows a section through a dorsal explant from an XFD mRNA injected embryo. **C** shows a section through a control lateral quadrant explant. **D** shows a section through a lateral explant from an XFD mRNA injected embryo. **E** shows a section through a control ventral quadrant explant. **F** shows a section through a ventral explant from an XFD mRNA injected embryo.

Fig. 7. Histology of dorsal, lateral and ventral explants taken from gastrula stage embryos injected with dominant negative FGF receptor (XFD) mRNA.



gastrulation there are a great many patterning events occurring that involve signals produced by cells on both the dorsal and ventral sides of the embryos (reviewed by Kimelman *et al.*, 1992; Sive, 1993; Slack, 1994). Therefore the characteristic whole embryo phenotype of XFD embryos may in part be due to atypical tissue interactions that are brought about by these aberrant cell movements. This must necessarily complicate the issue of interpreting the effects of inhibiting FGF activity on the formation of particular tissue types in the intact embryo. In order to circumvent this problem a comparison of the specification of dorsal, lateral and ventral embryo quarters from early gastrulae was undertaken. Embryos were injected into all blastomeres at the 4-cell stage and cultured until control stage 10.5. Explants were taken early in gastrulation before the aberrant dorsal cell movements drive extension around the lateral and ventral margins of the blastopore. Explants were cultured until control stage 41 and scored for the presence or absence of a particular tissue type. Therefore the figures presented in Table 2 represent the percentage of explants containing a given tissue type and therefore gives no indication of the volumes of each type in an explant. However, the figures do give an indication of the change in specification that results from inhibition of the FGF signalling pathway.

In dorsal explants there is only a small effect on the occurrence of cement gland and neural tissue. This is in keeping with rather normal anterior development in the whole embryo XFD phenotype. However, there is a dramatic reduction on both the frequency and volumes of notochord and muscle (Table 2, Fig. 7A and B). At the same time the presence of kidney, trunk mesenchyme and mesothelium was detected in a small number of XFD injected explants indicating

that there is a shift to more ventral-type specification. This ventralisation is also apparent in lateral explants from XFD injected embryos. It can be seen that there is a reduction in the occurrence of neural tissue, notochord, muscle and kidney (Table 2, Fig. 7C and D). Whereas there is an increase in the occurrence of the more ventral-type tissues mesothelium and blood. In ventral explants there is a reduction in the frequency of trunk mesenchyme and mesothelium. However, interestingly there does not seem to be much effect of FGF inhibition on either the frequency or amounts of the blood formed (Table 2, Fig. 7E and F).

An examination of the dominant negative FGF receptor phenotype clearly shows that there is a reduction in the total amount of mesoderm formed. The results on the specification of gastrula stage explants from XFD injected embryos are in keeping with those obtained by examining the whole embryo phenotype and further indicate that there is not only a reduction in the amount of mesoderm but also an alteration in its pattern. Thus much of the aberrant mesodermal pattern in the whole embryo can be accounted for by an effect on the specification of the mesoderm at the start of gastrulation.

Table 2 Effects of XFD mRNA injection on tissue differentiation in dorsal, lateral and ventral explants from gastrula stage embryos.

Explant	Cement gland (%)	Eye (%)	Other neural (%)	Notochord (%)	Muscle (%)	Kidney (%)	Mesenchyme (%)	Mesothelium (%)	Blood (%)	*n*
Dorsal										
Uninjected	100	90	100	100	100	0	0	0	0	10
XFD	81	75	94	56	31	6	12	6	0	16
Lateral										
Uninjected	0	0	78	44	78	67	89	56	22	9
XFD	0	0	14	0	36	21	57	71	86	14
Ventral										
Uninjected	0	0	0	0	0	0	100	100	100	10
XFD	0	0	0	0	0	0	57	86	100	14

XFD is the dominant negative FGF receptor. All blastomeres at the 4-cell stage were injected with 1 ng of the dominant negative FGF receptor (XFD). Embryos were cultured to the early gastrula stage 10.5 and were dissected into dorsal,lateral and ventral quarters. The explants were then cultured in isolation until control stage 41 and scored by histology for the presence of different tissue types. The score is based simply on the presence or absence of a particular tissue type in a given explant.

Properties of the organiser from XFD injected embryos

As has already been discussed the inhibition of FGF signalling clearly has a potent effect on the behaviour and differentiation of cells derived from the dorsal marginal zone. It is this region of the embryo that has the properties of the 'organiser'. Spemann and co-workers demonstrated that the dorsal lip of gastrula stage amphibian embryos when transplanted into an ectopic site of a host embryo has the ability to 'organiser' the formation of a secondary axis. Importantly it has been shown that the secondary axis contains tissues that have been recruited from the host embryo (Smith *et al.*, 1985). It is conceivable that interference with the properties of the organiser underlies some aspects of the XFD phenotype. This possibility has been investigated by ventral grafting and Einsteck-grafting of dorsal lip explants from XFD injected embryos into uninjected host embryos. The results from this experiment are shown in Table 3.

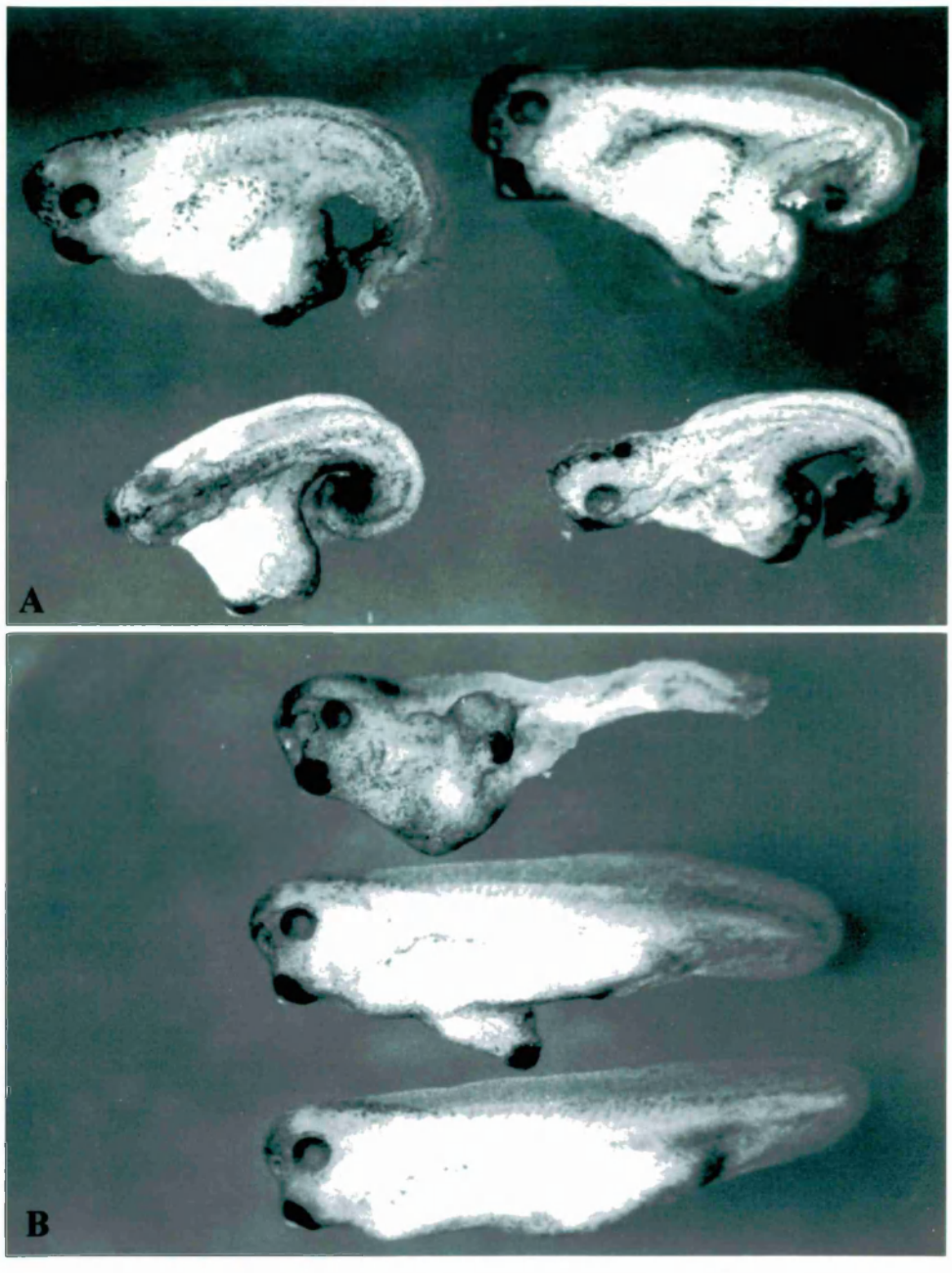
Table 3 Induction of secondary axes by dorsal lip grafts from embryos injected with dominant negative FGF receptor (XFD) mRNA.

Graft	Phenotype	*n*
Uninjected DMZ	7x twinned embryos with separate well formed heads 5x secondary anterior structures fused ventrally with host head	12
XFD injected DMZ	2x twinned embryos with separate well formed heads 5x secondary anterior structures fused ventrally with host head 2x posterior protrusion 1x no visible secondary axis	11

Gastrula stage 10.5 dorsal lip explants from uninjected control embryos or from embryos injected with 0.5 ng of XFD mRNA into all blastomeres at the 4-cell stage were either grafted into the blastocoel or on to the ventral side of stage 10.5 host embryos. Embryos were cultured until tailbud stage 35 and scored.

Fig. 8A and B show the results of organiser grafts. The grafts from normal embryos have produced very good secondary axes with well formed head and trunk structures. In contrast the secondary axes produced by the XFD grafts are much less complete. The head is less well formed and trunk structures are absent. These results suggests that the organising activity of the dorsal marginal zone is compromised by the inhibition of FGF signalling.

Fig. 8. Organiser grafts from embryos injected with dominant negative FGF receptor (XFD) mRNA.



Dorsal lip explants were taken at gastrula stage 10 from uninjected controls and embryos injected with 1 ng of XFD mRNA into each blastomere of the 4-cell stage. Explants were grafted into the ventral marginal zone of stage 10 uninjected host embryos and cultured until tailbud stage 35. **A** shows embryos resulting from control dorsal lip grafts. **B** shows embryos resulting from dorsal lip grafts from XFD mRNA injected embryos.

Discussion

The dominant negative FGF receptor (XFD) phenotype

Data in this chapter confirms the previous findings of (Amaya *et al.*, 1991; MacNicol *et al.*, 1993) and show that the phenotype of embryos, in which the FGF signal transduction pathway has been inhibited, is characterised by the complete absence of tail development. Trunk structures are either severely reduced or completely absent. Generally the morphology of anterior structures is much less severely affected. Essentially the whole of this phenotype can be recapitulated by injections of XFD mRNA into the dorsal half of the embryo. The phenotype produced by injection of XFD mRNA into the ventral half of the embryo is much less severe. In these embryos head and trunk structure are relatively normal. The development of the tail however, is greatly inhibited.

Cell movements in XFD embryos

Data in this chapter reveals that there is a sensitive requirement for FGF signalling on the dorsal side of the embryo. In XFD injected embryos, although there is some mesodermal ingression at the dorsal lip, which contributes to the relatively normal anterior structures seen in most XFD embryos, much of the dorsal tissues, which normally contribute to axial structures, fail to involute and extend along the developing axis. It appears that the characteristic open blastopore of XFD embryos results from the aberrant movement of these dorsal tissues into more lateral and ventral positions. The cellular basis for this phenomenon is unclear, but it is perhaps significant that the cells which contribute to the mesendoderm of the head in normal development and the head of XFD embryos, exhibit an active

crawling-type behaviour. This is quite different from the bipolar protrusive activity of axial mesoderm and neurectoderm cells, which drives mediolateral cellular intercalation and extension of dorsal midline structures along the developing anteroposterior axis (Keller *et al.*, 1992a; Keller *et al.*, 1992b). In XFD embryos there is a net movement of tissue from the dorsal side to more lateral and ventral positions. It is possible that the dorsal axial tissue is still undergoing cellular intercalation but, in contrast to normal development, leads to the narrowing and extension of dorsal tissues but in the wrong direction. In this view FGF signalling in the dorsal midline might be required as some kind of chemotactic agent influencing the direction in which extension normally occurs. It will require further work to determine the exact nature for the requirement of FGF signalling directing cell movement within the dorsal axis.

Effects of FGF inhibition on mesodermal differentiation

The demonstration of these abnormal cell movements is significant because it suggests that at least some of the XFD phenotype might result from the abnormal juxtapositioning of cell types. This might cause a failure of normal cellular interactions resulting in the loss of specific tissue types. Thus the loss of dorsal mesodermal tissues, such as the notochord and somite, in XFD injected embryos might result from an inhibitory effect from ventral and lateral tissues acting on the dorsal tissues which have moved into more lateral positions. Such inhibitory influences on dorsal development have been proposed to be important in determining the relative sizes of the dorsal and ventral territories during normal development (Kimelman *et al.*, 1992; Jones, 1996)

A comparison of the histology of whole embryos injected with XFD mRNA and explants, that were removed from XFD embryos at the start of gastrulation before the aberrant cell movements take place, is useful in determining the relative importance of cell movements in the development of the XFD phenotype. The results obtained from whole embryos and explant culture are in quite good agreement and suggest that there is a requirement for FGF signalling in the development of most mesodermal tissue types including both dorsal tissues, such as notochord and muscle, and more ventral tissue-types such as kidney, trunk mesenchyme and mesothelium. The one exception to this is blood, which, to a large extent, appears not to require a functional FGF signalling pathway for its differentiation. The broad similarity in results obtained in whole embryo and explant culture suggests that much of the effect on tissue differentiation following FGF is largely independent of aberrant cell movements.

Histology indicates that inhibition of FGF function during early development does result in a reduction in the amount of mesoderm formation. The presence of thickened patches of atypical epidermis in XFD embryos suggests that there is a corresponding increase in the the amount of ectodermal derivatives. These results are consistent with the view that the FGFs are required for the initial stages of mesoderm formation but are by no means conclusive. The terminal differentiation of specific tissue types occurs long after the initial events required for their specification. It is possible that the failure of mesoderm formation caused by injection of XFD mRNA may result from an interference with subsequent differentiation events.

Effects of FGF inhibition on anteroposterior (AP) and dorsoventral (DV) patterning

It can be said with some certainty that the FGFs are not required for specification of anterior structures because they are relatively unaffected in XFD embryos. A striking feature of the XFD phenotype is the loss of posterior structures. As already discussed above, the interpretation of how the loss of structures arises is complicated by the possible involvement of the FGFs in cell differentiation.

The Spemann organiser is intimately involved in the development of the anteroposterior axis (reviewed by Slack and Tannahill, 1992). Data in this chapter shows that the Spemann organiser from XFD embryos is compromised in its ability to induce a secondary axis in a normal host embryo. This effect on the activity of the organiser is likely to account for at least some of the effects on AP pattern in XFD embryos. Furthermore, the increase in ventral tissues in dorsal and lateral explants from XFD embryos may be due to an interference with the dorsalising activity of the organiser (reviewed by Sive, 1993).

Experiments in this chapter clearly demonstrate that the activity of the FGFs is in some way required for the formation of the mesoderm. Experiments in the next chapter seek to address the issue of when during development the activity of the FGFs is required.

Chapter 6

Gene expression in dominant negative FGF receptor injected embryos.

Introduction

As has already been discussed, injection of mRNA coding for the dominant negative FGF receptor (XFD) provides a powerful tool for the analysis of the role that FGF signalling has during the early development of *Xenopus laevis.* The injection of XFD mRNA has a number of consequences. The previous chapter showed that the inhibition of the FGF signal transduction pathway in early development of results in specific phenotype that is characterised by a pertubation of normal morphogenetic movements during gastrulation and a derangement of the normal pattern of both the dorsoventral and anteroposterior axes. Most dramatically there is an almost complete loss of trunk and posterior structures. However, an examination of the gross phenotypic changes provides very little information on the underlying molecular processes which require the activity of the FGFs. Moreover, the differentiation of specific tissue types is likely to involve many cellular interactions subsequent to the initial specification event. Hence the characteristic effects on tissue differentiation seen in XFD injected embryos give very little indication as to the period in which FGF signalling is required.

In recent years a large number of both regional and tissue specific molecular markers from *Xenopus* have become available. This chapter contains a detailed analysis of the effects of inhibiting the FGF signal transduction pathway on the expression a wide range of regional and tissue specific markers by RNAase protection and *in situ* hybridisation through gastrula and neurula stages. RNAase protection provides a quantitative assessment on the effects on gene expression, whereas *in situ* hybridisation allows the analysis of effects on gene expression within particular regions of the developing embryo. Attention was

focused on the examination of gene expression within the gastrula/neurula period because it was thought that this would provide useful information not only on the role that the FGFs might have in mesoderm induction during the blastula period but also on the involvement of FGF signalling in subsequent patterning events that lead to the establishment of the major body axes (Slack *et al.*, 1992; Sive, 1993).

Data in this chapter indicate that the FGFs are not only required for mesoderm induction but are also likely to be involved in regulating gene expression within the mesoderm in the gastrula/neurula period. These experiments help define a subset of genes, whose expression requires FGF signalling. Prominent amongst the genes expressed in the mesoderm that require the activity of the FGFs are *Xbra* and the myogenic basic helix-loop-helix (bHLH) *XmyoD* and *Xmyf5*.

Interestingly the onset of transcription from *HoxA7* (*Xhox36*) and *HoxC6* (*Xlhbox1*) is delayed in XFD injected embryos. The Hox genes are expressed in both mesoderm and ectoderm lineages, indicating an additional requirement for FGF activity in patterning of the ectoderm.

Materials and methods

RNAase protection analysis of gene expression in XFD injected embryos

RNA injections, RNA isolation and RNAase protection analyses were carried out as per general methods. Autoradiography exposures were from 1 to 10 days. Embryos were injected with 1 ng of each mRNA into each blastomere at the 4-cell stage and allowed to develop until the required stage. The data presented here represents the results from one experiment on the same batch of embryos. For

each experimental group (uninjected, d50 injected and XFD injected) 10 embryos were collected for each time point (stage 10, 13 and 17).

In situ hybridisation analysis of gene expression in XFD injected embryos

In situ hybridisations were carried out on albino embryos as described in general methods. For each stage and probe at least 10 embryos were processed. Photographs are of typical embryos for a particular marker analysed. Embryos were injected with 1 ng of XFD mRNA into each blastomere at the 4-cell stage and allowed to develop until the required stage.

Results

Analysis of mesodermal gene expression in XFD injected embryos

Embryos in which the FGF signal transduction pathway has been inhibited show abnormalities in both the quantity and pattern of the mesoderm. In order to gain a better understanding of the requirement for FGF signalling in formation of the mesoderm the expression of a number of molecular markers in XFD injected embryos was analysed by both RNAase protection and *in situ* hybridisation

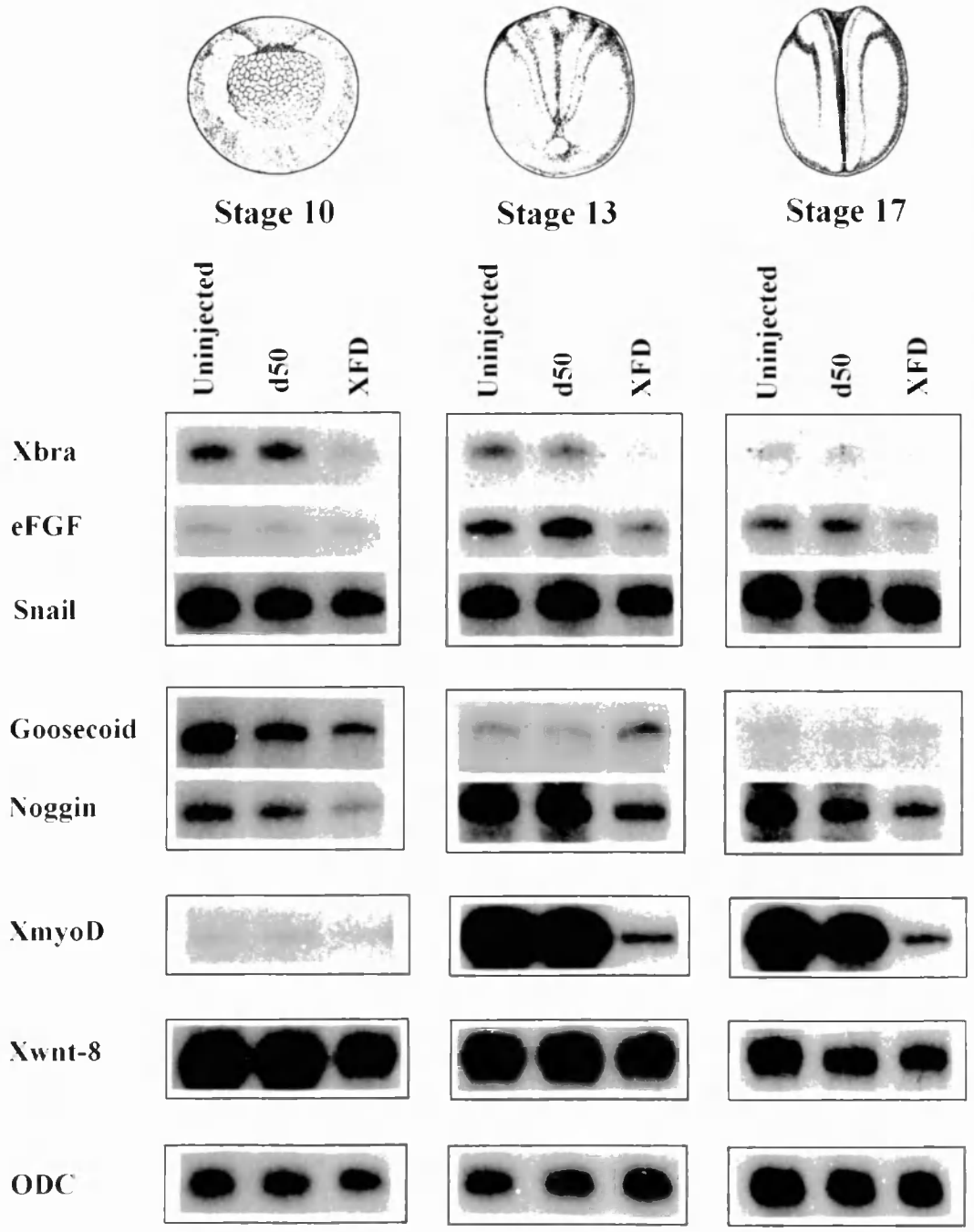
The general mesodermal markers, Xbra, Xsna and eFGF

Fig. 1 shows that in XFD injected embryos expression of the general mesodermal marker *Xbra* is greatly down regulated compared to controls at the start of gastrulation. This level of expression continues to fall through gastrula and neurula stages. Fig. 2 A and B are *in situs* of gastrula stage 11 embryos showing the almost complete absence of *Xbra* expression in an XFD injected embryo.

Fig. 1. RNAase protection analysis of mesodermal marker gene expression in embryos injected with dominant negative FGF receptor (XFD) mRNA.

Control uninjected embryos and embryos injected with either 1 ng of receptor control (d50) mRNA or 1 ng of dominant negative FGF (XFD) receptor mRNA into each blastomere at the 4-cell stage were cultured until early gastrula stage 10, late gastrula stage 13 and late neurula stage 17. 5 μg of total RNA from each stage was analysed by RNAase protection for expression of the above panel of marker genes. All assays shown were carried out on RNA from the same experiment. The *ODC* loading control shown is a representative example.

Fig. 1. RNAase protection analysis of mesodermal marker gene expression in embryos injected with dominant negative FGF receptor (XFD) mRNA.



The zinc finger transcription factor *Xsna* is also expressed in the mesoderm of the blastopore at the start of gastrulation (Essex *et al.*, 1993) but unlike *Xbra* the overall levels of *Xsna* expression is not significantly affected by inhibition of the FGF signalling pathway (Fig. 1). This is confirmed by *in situ* hybridisation (Fig. 2C and D). However, later in gastrulation *Xsna* expression is detected in the presumptive neural crest forming region of the ectoderm (see Fig. 2E) (Essex *et al.*, 1993). This ectodermal expression of *Xsna* is completely eliminated in XFD injected embryos (Fig. 2F).

At the start of gastrulation the level of *eFGF* expression is not greatly affected by the inhibition of FGF signalling. However, in the late gastrula and neurula the level of *eFGF* expression in XFD embryos is depressed relative to controls (Fig. 1). Fig. 2G and H. show *eFGF* expression is still present in the periblastopore region of XFD injected embryos at stage 11.5, although at a somewhat lower level than in controls.

The dorsal mesodermal markers, goosecoid and noggin

Goosecoid is a homeobox containing gene which has been implicated in the formation and activity of the Spemann organiser (Cho *et al.*, 1991). It is expressed in the deep mesendodermal tissue layer of the dorsal blastopore lip at the start of gastrulation in cells which are fated to form pharyngeal endoderm, head mesoderm and possibly some anterior notochord (De Robertis *et al.*, 1992; Vodicka and Gerhart, 1995). The expression of *goosecoid* is not significantly affected in XFD injected embryos (Fig. 1).

Noggin encodes a secreted factor that has also been implicated in the activity of the organiser. It is able to induce neural tissue in ectoderm and to dorsalise ventral mesoderm (Lamb *et al.*, 1993; Smith *et al.*, 1993). *Noggin* is initially expressed at the start of gastrulation in the dorsal lip and later along the length of the notochord (Smith and Harland, 1992). The overall level of *noggin* expression is somewhat down regulated in XFD embryos throughout the gastrula/neurula period (Fig.1). Fig 2I and J show *in situ* hybridisations of *noggin* in gastrula stage 11.5 embryos. *Noggin* expression can be seen in the developing notochord of the control embryo. Consistent with the RNAase protection data, the level of *noggin* expression is lower in XFD embryos. The expression seen in these embryos is very diffuse and is barely detectable in the region of the dorsal lip.

The *ventrolateral mesodermal marker Xwnt-8*

Xwnt-8 is a member of the Wnt family of secreted proteins. Several wnts have are expressed in the early development of *Xenopus* (Christian *et al.*, 1991a; Christian *et al.*, 1991b; Ku and Melton, 1993) and have been shown to have a number of biological activities including the ability to rescue axial development in UV ventralised embryos (Smith and Harland, 1991). Indeed there is a considerable body of evidence that suggests that a wnt family member is involved in the induction and subsequent activities of the Spemann organiser (Heasman *et al.*, 1994; Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995). While Xwnt-8 does exhibit axis rescuing activity it is very unlikely to be required for normal axial development because during normal development it is not expressed

Fig. 2. Whole-mount *in situ* hybridisation analysis of mesodermal marker gene expression in embryos injected with dominant negative FGF receptor (XFD) mRNA.

Abbreviations: **dl**=dorsal blastopore lip. **ntc**=notochord.

Control uninjected embryos and embryos injected with either 1 ng of dominant negative FGF (XFD) receptor mRNA into each blastomere at the 4-cell stage were cultured until the relevant stage.

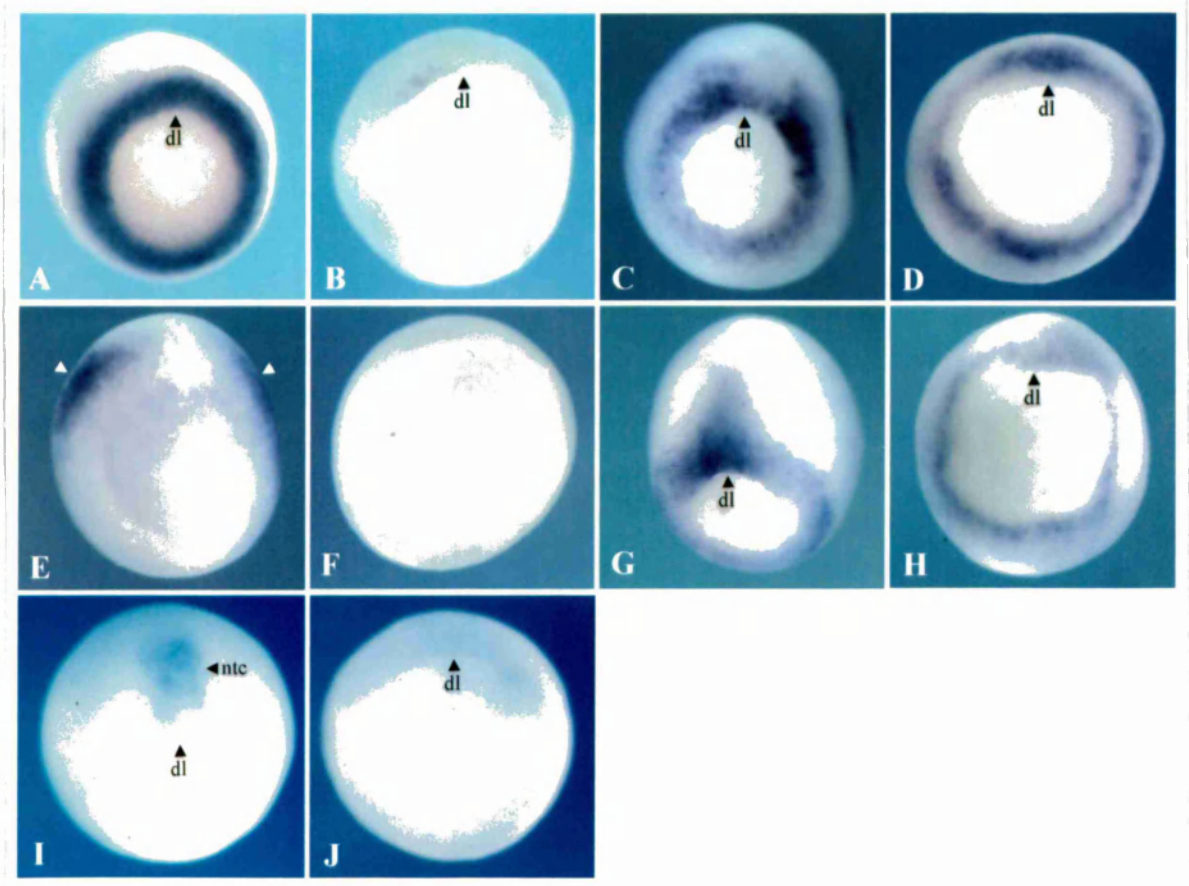
A shows a vegetal view of a control gastrula stage 11 embryo hybridised with a DIG labelled *Xbra* antisense probe (dorsal to the top). **B** shows a vegetal view of an XFD mRNA injected gastrula stage 11 embryo hybridised with a DIG labelled *Xbra* antisense probe (dorsal to the top).

C shows a vegetal view of a control gastrula stage 11.5 embryo hybridised with a DIG labelled *Xsna* antisense probe (dorsal to the top). **D** shows a vegetal view of an XFD mRNA injected gastrula stage 11.5 embryo hybridised with a DIG labelled *Xsna* antisense probe (dorsal to the top). **E** shows an animal view of a control gastrula stage 11.5 embryo hybridised with a DIG labelled *Xsna* antisense probe (dorsal to the top). White arrows indicate staining in the presumptive neural crest forming regions. **F** shows an animal view of an XFD mRNA injected stage 11.5 embryo hybridised with a DIG labelled *Xsna* antisense probe (dorsal to the top).

G shows a dorsovegetal view of a control gastrula stage 11.5 embryo hybridised with a DIG labelled *eFGF* antisense probe (dorsal to the top). **H** shows a vegetal view of an XFD mRNA injected gastrula stage 11.5 embryo hybridised with a DIG labelled *eFGF* antisense probe (dorsal to the top).

I shows a dorsovegetal view of a control gastrula stage 11.5 embryo hybridised with a DIG labelled *noggin* antisense probe (dorsal to the top). **J** shows a vegetal view of an XFD mRNA injected gastrula stage 11.5 embryo hybridised with a DIG labelled *noggin* antisense probe (dorsal to the top).

Fig. 2. Whole-mount *in situ* hybridisation analysis of mesodermal marker gene expression in embryos injected with dominant negative FGF receptor (XFD) mRNA.



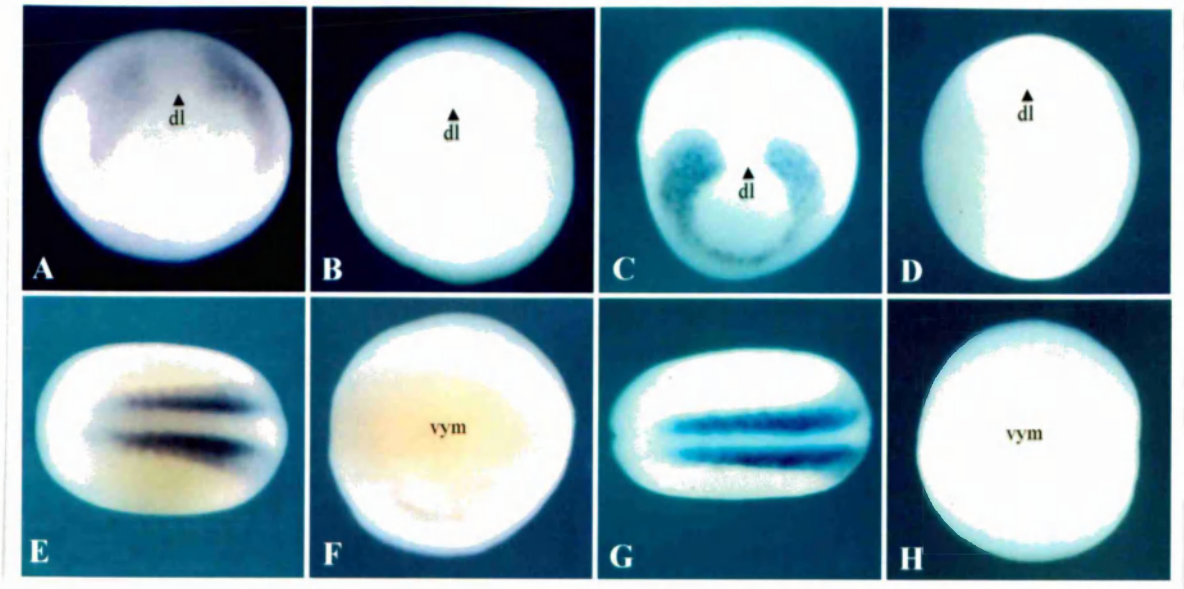
in dorsal regions and is only expressed in the mesoderm and endoderm of the lateral and ventral marginal zone and vegetal hemisphere during gastrula stages (Smith and Harland, 1991; Lemaire and Gurdon, 1994). Fig. 1 shows that there is very little effect on the expression of *Xwnt-8* in XFD injected embryos.

The myogenic markers, XmyoD, Xmyf5 and muscle actin

The basic helix-loop-helix (bHLH) proteins XmyoD has been implicated in the development of muscle lineages in a wide range of organisms from *C.elegans* to mouse (Weintraub, 1993; Rudnicki and Jaenisch, 1995). In *Xenopus* the development of *XmyoD* expression is rather complex. At the MBT there is a low level ubiquitous activation of expression. During gastrula stages expression increases enormously and becomes localised to the region of the embryo that will contribute to the muscle lineages (Frank and Harland, 1991; Harvey, 1991). The expression of *XmyoD* and *Xmyf5* is specifically excluded from region of the dorsal organiser (Fig. 3A and C) (Hopwood *et al.*, 1989). Interestingly there is no effect in XFD embryos on the initial low level of *XmyoD* expression detected at the start of gastrulation. However, later in gastrulation and neurula stages there is an enormous reduction in the levels of *XmyoD* expression compared to controls (Fig. 1).

Inhibition of FGF function clearly has a dramatic effect on the expression of *XmyoD*, therefore it was decided to examine the effects of XFD injection on the expression of another myogenic bHLH gene *Xmyf5* (Hopwood *et al.*, 1991) and cardiac *actin*, which is a marker of muscle terminal differentiation (Mohun *et al.*, 1984). At mid-gastrula stage 11 the expression of *XmyoD* and *Xmyf5* is not

Fig. 3. Whole-mount *in situ* hybridisation analysis of myogenic gene expression in embryos injected with dominant negative FGF receptor (XFD) mRNA.



Abbreviations: **dl**=dorsal blastopore lip. **ntc**=notochord. **vym**=vegetal yolk mass.

Control uninjected embryos and embryos injected with either 1 ng of dominant negative FGF (XFD) receptor mRNA into each blastomere at the 4-cell stage were cultured until the relevant stage.

A shows a vegetal view of a control gastrula stage 11 embryo hybridised with a DIG labelled *XmyoD* antisense probe (dorsal to the top). **B** shows a vegetal view of an XFD mRNA injected gastrula stage 11 embryo hybridised with a DIG labelled *XmyoD* antisense probe (dorsal to the top).

C shows a vegetal view of a control gastrula stage 11 embryo hybridised with a DIG labelled *Xmyf5* antisense probe (dorsal to the top). **D** shows a vegetal view of an XFD mRNA injected gastrula stage 11 embryo hybridised with a DIG labelled *Xmyf5* antisense probe (dorsal to the top).

E shows a dorsal view of a control late neurula stage 20 embryo hybridised with a DIG labelled *XmyoD* antisense probe (anterior to the left). **F** shows a vegetal view of an XFD mRNA injected gastrula stage 20 embryo hybridised with a DIG labelled *XmyoD* antisense probe.

G shows a dorsal view of a control late neurula stage 20 embryo hybridised with a DIG labelled *actin* antisense probe (anterior to the left). **H** shows a vegetal view of an XFD mRNA injected gastrula stage 20 embryo hybridised with a DIG labelled *actin* antisense probe.

detectable by *in situ* hybridisation in XFD injected embryos (Fig. 3B and D). Expression of XmyoD is still completely suppressed in XFD embryos at stage 20 (Fig. 3E and F). Furthermore, Fig. 3G and H show that at stage 20 there is no detectable staining for cardiac actin in XFD embryos, indicating that the complete absence of differentiated skeletal muscle.

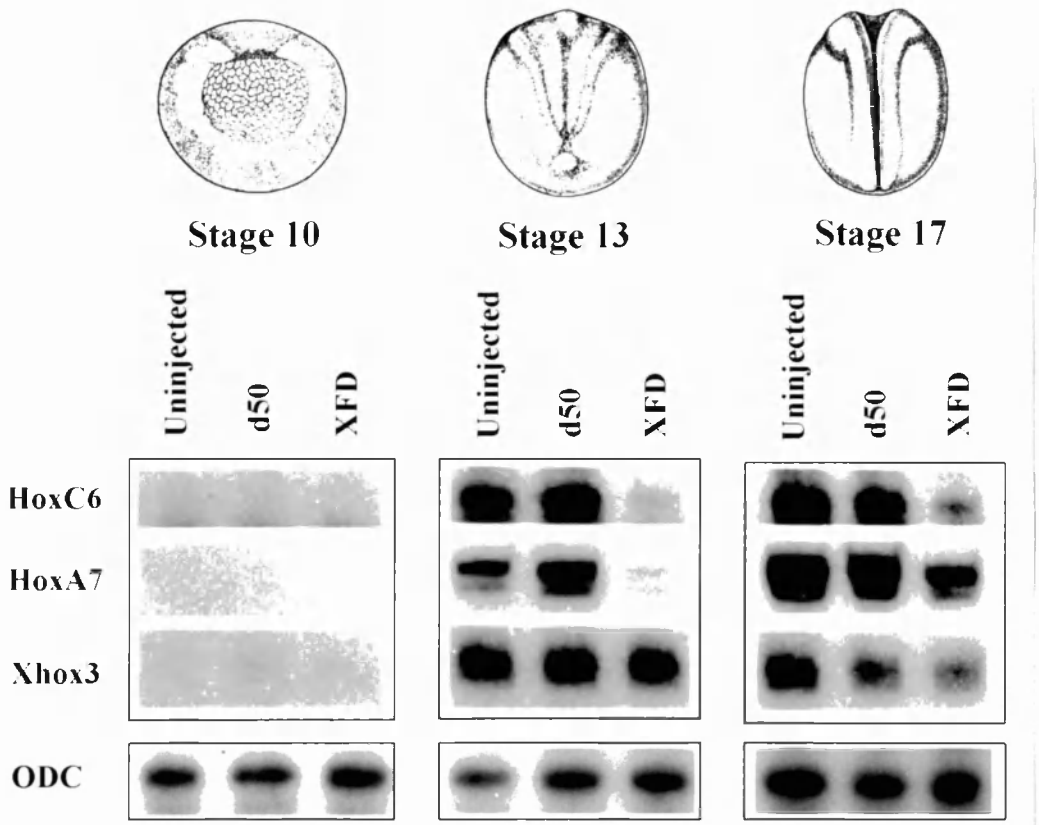
Analysis of anteroposterior marker gene expression in XFD injected embryos

Embryos overexpressing XFD show marked reductions in posterior structures. In order to understand better the requirement for FGF activity in posterior development the expression of a number of genes which are thought to be involved in anteroposterior specification was examined in XFD injected embryos.

The anteroposterior markers HoxB1, HoxC6, HoxA7 and Xhox3

It is now well established that genes of the vertebrate Hox clusters are directly involved in specification of antero-posterior levels along the body axis and so it is significant that activation of expression of the Hox cluster genes *HoxC6* (*XlHbox1*) and *HoxA7* (*Xhox36*) is delayed and greatly reduced in such embryos (Fig 4.). *HoxC6* has an anterior limit of expression in the spinal cord but is also expressed in the posterior mesoderm (Carrasco and Malacinski, 1987). *HoxA7* is also expressed in posterior ectoderm and mesoderm (Condie and Harland, 1987). These data suggest that the activity of the FGFs is required for the regulation of gene expression not only in the mesoderm but also in the ectoderm. Unlike the situation with *Xbra* and the myogenic genes it can be seen that the expression of

Fig. 4. RNAase protection analysis of anteroposterior marker gene expression in embryos injected with dominant negative FGF receptor (XFD) mRNA.



Control uninjected embryos and embryos injected with either 1 ng of receptor control (d50) mRNA or 1 ng of dominant negative FGF (XFD) receptor mRNA into each blastomere at the 4-cell stage were cultured until early gastrula stage 10, late gastrula stage 13 and late neurula stage 17. 5 µg of total RNA from each stage was analysed by RNAase protection for expression of the above panel of marker genes. All assays shown were carried out on RNA from the same experiment. The *ODC* loading control shown is a representative example.

the Hox genes begins to recover somewhat during neurula stages. This indicates that although in normal development *Hox* gene expression is activated during gastrula stages the factors that are responsible for this are present through neurula stages and continue to be able to activate *de novo* Hox gene. By the late neurula stage XFD mRNA is being degraded and it is to be expected that some FGF signalling will have recovered. It is possible that this accounts for the recovery of Hox gene expression at this stage.

The homeobox containing gene *Xhox3,* which is the *Xenopus* homologue of the murine *Evx 1* gene, has been implicated in the specification of posterior structures in *Xenopus* and zebrafish (Ruiz i Altaba and Melton, 1989c; Barro *et al.*, 1994). Moreover, animal caps treated with FGF strongly express *Xhox3* and in normal development *Xhox3* is expressed in a posterior to anterior gradient within the mesoderm during gastrula stages (Ruiz i Altaba and Melton, 1989a; Ruiz i Altaba and Melton, 1989b). Therefore it is perhaps surprising to find that in XFD embryos, which exhibit a massive reduction in posterior development, there is no effect on the expression of *Xhox3* (Fig. 4).

The effects of FGF inhibition on Hox gene expression have been investigated further using *in situ* hybridisation to *HoxA7* and *HoxB1*. Fig. 5A shows the normal expression of *HoxA7* in this dorsal view of a late gastrula stage 13 embryo. Staining is seen in the posterior of the embryo around the closed blastopore and at this stage is absent from the dorsal midline. Fig. 5B confirms that at this stage *HoxA7* expression is completely abolished in XFD embryos. However, in the late neurula stage 20 there is some recovery of expression around the open blastopore of XFD embryos (Fig. 5C and D).

Fig. 5. Whole-mount *in situ* hybridisation analysis of anteroposterior marker gene expression in embryos injected with dominant negative FGF receptor (XFD) mRNA.

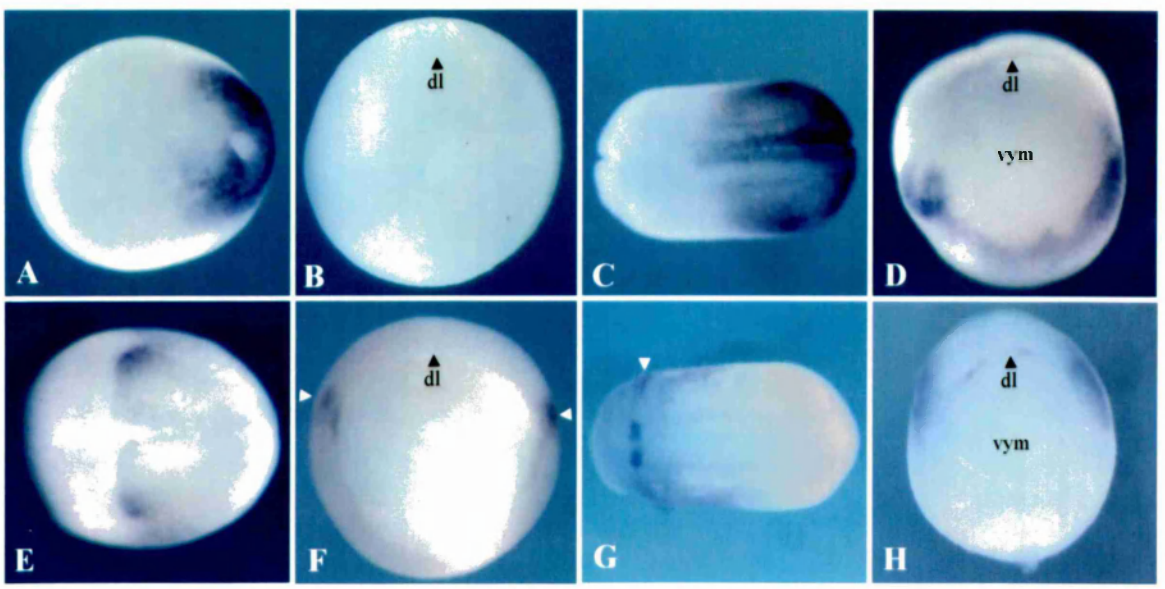
Abbreviations: **dl**=dorsal lip. **vym**=vegetal yolk mass

Control uninjected embryos and embryos injected with either 1 ng of dominant negative FGF (XFD) receptor mRNA into each blastomere at the 4-cell stage were cultured until the relevant stage.

A shows a dorsal view of a control late gastrula/early neurula stage 13 embryo hybridised with a DIG labelled *HoxA7* antisense probe (anterior to the left). **B** shows a vegetal view of an XFD mRNA injected gastrula stage 13 embryo hybridised with a DIG labelled *HoxA7* antisense probe (dorsal to the top). **C** shows a dorsal view of a control stage 20 embryo hybridised with a DIG labelled *HoxA7* antisense probe (anterior to the left). **D** shows a vegetal view of an XFD mRNA injected stage 20 embryo hybridised with a DIG labelled *HoxA7* antisense probe (dorsal to the top).

E shows a dorsal view of a control stage 13 embryo hybridised with a DIG labelled *HoxB1* antisense probe (anterior to the left). **F** shows a vegetal view of an XFD mRNA injected stage 13 embryo hybridised with a DIG labelled *HoxB1* antisense probe (dorsal to the top). White arrows indicate expression domain split around the open blastopore. **G** shows a dorsal view of a control stage 20 embryo hybridised with a DIG labelled *HoxB1* antisense probe (anterior to the left). **H** shows a vegetal view of an XFD mRNA injected stage 20 embryo hybridised with a DIG labelled *HoxB1* antisense probe (dorsal to the top. White arrow indicates expression in neural crest streaming out of the hindbrain.

Fig. 5. Whole-mount *in situ* hybridisation analysis of anteroposterior marker gene expression in embryos injected with dominant negative FGF receptor (XFD) mRNA.



HoxB1 is one of the most anteriorally expressed Hox genes, which is expressed as a stripe in the developing hindbrain (Godsave *et al.*, 1994) and as such provides a useful comparison to the more posteriorally expressed *HoxA7*. Fig. 5E shows the normal expression pattern of *HoxB1* in this dorsal view of a stage 13 embryo. The vegetal view of an XFD embryo from the same stage shows that *HoxB1* is still expressed but the aberrant dorsal cell movements of the XFD embryo have resulted in the normal single stripe of expression being spread laterally around the open blastopore. In the late neurula stage 20 the *HoxB1* domain in control embryos is restricted to a narrow stripe in the hindbrain and neural crest emerging from the hindbrain (Fig. 5G). In XFD embryos the 2 *HoxB1* stripes are still present in their lateral position around the open blastopore but unlike the wild-type stripes are very much more diffuse (Fig. 5H). These results indicate that the signals required for the activation of the anteriorally expressed *HoxB1* gene are still present in XFD embryos. These data suggest that there are 2 subgoups of Hox genes which can be classified according their sensitivity to the inhibition of FGF signalling. The normal activation of expression from the posterior Hox genes, such as *HoxA7* and *HoxC6*, is FGF dependent, whereas activation of the anterior Hox genes such as *HoxB1* is not FGF dependent. This result may be significant given the dramatic posterior reductions and relatively normal anterior development which characterises the dominant negative FGF receptor phenotype.

Analysis of anterior neural gene expression in XFD injected embryos

Although the spatial expression of the anterior neural marker *HoxB1* is somewhat abnormal in XFD embryos the overall level of expression is not dramatically affected. This is in contrast with the effect on more posterior Hox genes, such as *HoxA7* and *HoxC6*, in XFD injected embryos. The rather normal anterior development in XFD embryos might suggest that this likely to be the case for most anteriorally expressed genes. This has been investigated with using the markers *otx2*, *en-2* and *krox20*, which are markers of the forebrain, midbrain/hindbrain junction and hindbrain respectively (Hemmati-Brivanlou and Harland, 1989; Bradley *et al.*, 1992; Pannese *et al.*, 1995).

The general neural marker NCAM

The spatial extent of neural development was examined in XFD injected embryos by looking at the expression of the neural adhesion molecule *NCAM* (Krieg *et al.*, 1989). The *in situ* hybridisations in Fig. 6 and B show that the overall levels of *NCAM* expression are considerably reduced in XFD injected late neurula stage 20 embryos. *NCAM* expression is seen on the dorsal side of the embryo close to the blastopore where the head will form. However, interestingly there is also some NCAM staining around the margin of the open blastopore indicating that the aberrantly spread dorsal mesoderm still retains the ability to induce the expression of, at least some, neural markers.

Fig. 6. Whole-mount *in situ* hybridisation analysis of anterior neural marker gene expression in embryos injected with XFD mRNA.

Abbreviations: **dl**=dorsal lip. **vym**=vegetal yolk mass.

Control uninjected embryos and embryos injected with either 1 ng of dominant negative FGF (XFD) receptor mRNA into each blastomere at the 4-cell stage were cultured until the relevant stage.

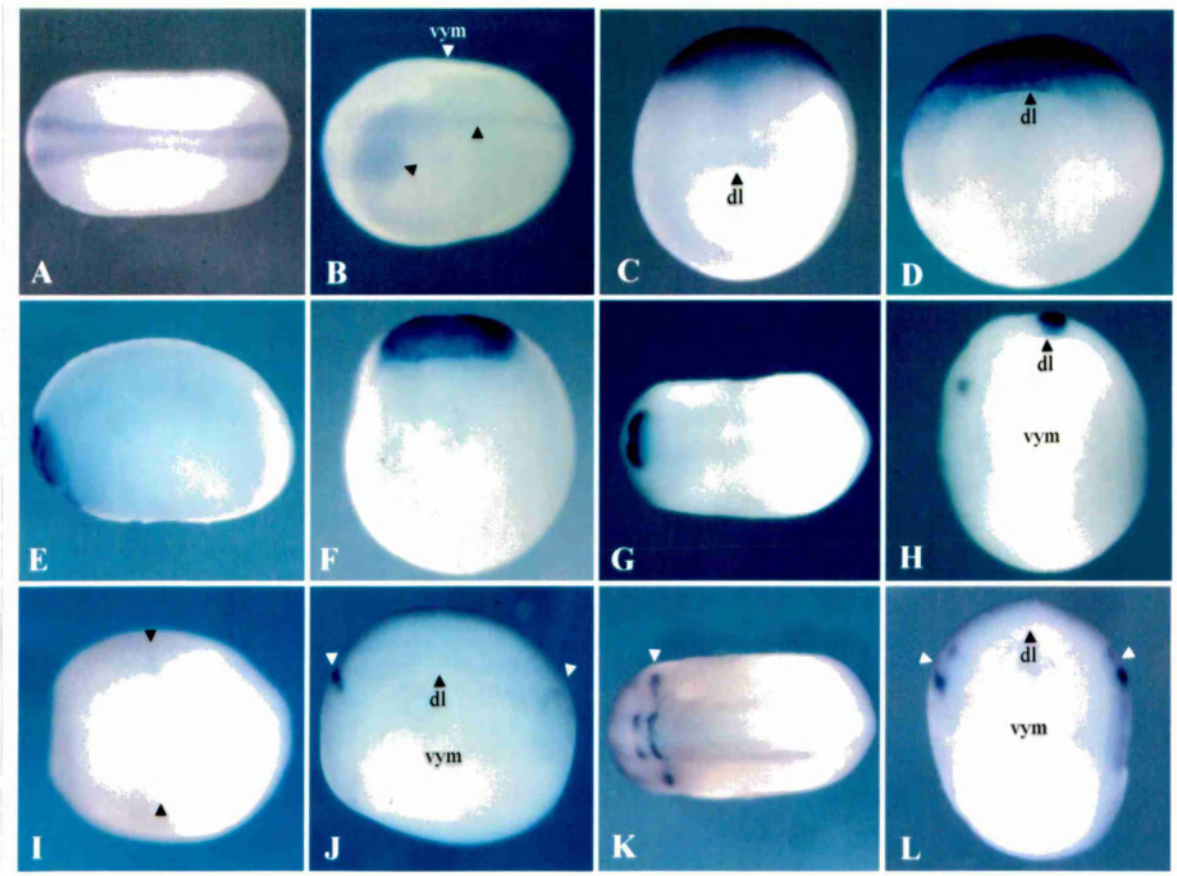
A shows a dorsal view of a control stage 20 embryo hybridised with a DIG labelled *NCAM* antisense probe (anterior to the left). **B** shows a side view of an XFD mRNA injected stage 20 embryo hybridised with a DIG labelled *NCAM* antisense probe (vegetal to the top, dorsal lip to the left). White arrow indicates the exposed vegetal yolk mass.

C shows an dorsovegetal view of a control gastrula stage 11.5 embryo hybridised with a DIG labelled *otx2* antisense probe (dorsal to the top). **D** shows an vegetal view of an XFD mRNA injected stage 11.5 embryo hybridised with a DIG labelled *otx2* antisense probe (dorsal to the top). **E** shows a side view of a control stage 20 embryo hybridised with a DIG labelled *otx2* antisense probe (anterior to the left, dorsal to the top). **F** shows an animal view of an XFD mRNA injected stage 20 embryo hybridised with a DIG labelled *otx2* antisense probe (dorsal to the top, exposed yolk mass into the page.

G shows a dorsal view of a control stage 20 embryo hybridised with a DIG labelled *en-2* antisense probe (anterior to the left). **H** shows a vegetal view of an XFD mRNA injected stage 20 embryo hybridised with a DIG labelled *en-2* antisense probe (dorsal to the top).

I shows a dorsal view of a control late gastrula/early neurula stage 13 embryo hybridised with a DIG labelled *krox20* antisense probe (anterior to the left). Black arrows indicate faint stripe of expression in neural plate. **J** shows a vegetal view of an XFD mRNA injected gastrula stage 13 embryo hybridised with a DIG labelled *krox20* antisense probe (dorsal to the top). White arrows indicate stripes of expression split around the open blastopore. **K** shows a dorsal view of a control stage 20 embryo hybridised with a DIG labelled *krox20* antisense probe (anterior to the left). **L** shows a vegetal view of an XFD mRNA injected stage 20 embryo hybridised with a DIG labelled *krox20* antisense probe.

Fig. 6. Whole-mount *in situ* hybridisation analysis of anterior neural marker gene expression in embryos injected with XFDmRNA.



The anterior neural markers otx2, en-2 and krox 20

Otx2 is a *Xenopus* homologue of the *orthodenticle* gene, which is involved in the development of head structures in *Drosophila* and has been widely implicated, along with the *emx* class of genes, in a having a similar role in the vertebrates (Holland *et al.*, 1992; Pannese *et al.*, 1995). At the start of gastrulation the initial expression of *otx2* is in the region of the organiser and as gastrulation proceeds the expression of *otx2* moves away from the region of the blastopore toward the future anterior of the embryo. At mid-gastrula stage 11.5 Fig. 6C shows that *otx2* expression is prominent in the anterior neural plate although expression is also found in the anterior mesendoderm. Fig. 6D shows that in XFD embryos of the same stage the levels of *otx2* expression appear to be quite normal but in keeping with previous results the domain expression remains close to the blastopore. Even at the late neurula stage 20, the anterior domain of *otx2* is comparable in XFD and control embryos (Fig. 6E and F).

The homeobox gene *en-2* is a homologue of the *Drosophila* segment polarity gene *engrailed* that provides a useful marker for the midbrain/hindbrain junction. *en-2* expression is not detectable by *in situ* hybridisation until the late neural stage when it is first apparent as single stripes either side of the dorsal midline in the anterior neural plate (Brivanlou and Harland, 1989). Fig. 6G shows the normal expression of *en-2* in a control embryo at stage 20. Fig. 6H shows *en-2* expression in an XFD embryo from the same stage. Note the high level of *en-2* expression close to blastopore at the dorsal midline and a spot of lower level expression some distance around the margin of the blastopore. This pattern is quite reproducible and may indicate that the *en-2* domain lies very close to the

anteroposterior level at which the axis splits in XFD embryos. This is further supported by the observation that the expression of the more posterior genes *HoxB1* and, as will now be discussed, *krox20* is characteristically split around the open blastopore in XFD embryos.

krox20 is a zinc finger gene which is believed to be involved in the assignment of rhombomere identity in the developing hindbrain. Fig. 6I shows that in control embryos *krox20* expression is first apparent at the early neurula stage 14 as a single stripe either side of the dorsal midline of the neural plate. Later in development two stripes of *krox20* expression are seen in the hindbrain in rhombomeres 3 and 5 (r3 and r5) and in the neural crest streaming out of r5 (Fig. 6K). As with *HoxB1*, the *krox20* stripes of expression are split around the open blastopore in XFD embryos suggesting the basic mechanism for the establishment of their expression is unaffected by FGF inhibition (Fig. 6J and L).

Discussion

The FGFs and mesoderm induction.

A number of genes have been identified which are activated in the newly induced mesoderm of the marginal zone (MZ) of the late blastula stage *Xenopus* embryo. The activation of expression from *Xbra* is an immediate early response to mesoderm induction by FGF-like and activin-like mesoderm inducing factors (Smith *et al.*, 1991). The induction of expression of the homeobox gene *goosecoid*, is also an immediate early response, but only to activin-like mesoderm inducing factors (Blumberg *et al.*, 1991). By definition this means that activation of their expression does not require protein synthesis. Therefore anything that

interferes with mesoderm induction by the maternal inducers, prior to the onset of zygotic transcription, should affect the expression of these immediate early markers. Data in this chapter and elsewhere shows that *Xbra* expression is down regulated in very late blastula/early gastrula stage embryos that have been injected with the dominant negative FGF receptor (XFD) mRNA (Amaya *et al.*, 1993; Isaacs *et al.*, 1994). This indicates that FGF function is required for mesoderm induction and that part of this requirement is likely to be maternal. However, as has already been discussed the known maternal FGFs are not vegetally localised as would be expected for one of the maternal inducers. The explanation of this is complex and reveals a previously unexpected link between the FGF and activin-like molecule signal transduction pathways.

The enzyme MAP kinase is a down stream element of the, now well characterised FGF tyrosine kinase signal transduction pathway. Treatment of blastula stage animal caps with FGF leads to a rapid phosphorylation and activation of MAP kinase (LaBonne and Whitman, 1994). The details of signal transduction from activin-like molecules is not as clearly understood as is the case for the FGFs. Unlike the FGF receptor, the activin receptor complex has serine/threonine kinase activity and treatment of animal caps with activin does not lead to a rapid phosphorylation of MAP kinase (LaBonne and Whitman, 1994). This strongly suggest that activin signalling does not use any of the elements of the FGFs signalling pathway upstream of MAP kinase. Paradoxically however, overexpression of inhibitory forms of these upstream elements, such as the FGF receptor (XFD), ras, raf or inhibition of MAP kinase itself by overexpression of a MAP kinase phosphatase blocks mesoderm induction and the immediate early

activation of *Xbra* by activin treatment (Whitman and Melton, 1992; LaBonne and Whitman, 1994; LaBonne *et al.*, 1995). In some way there is a concurrent requirement for activity of the FGF tyrosine kinase signal transduction pathway in animal caps that is necessary to allow activin to induce the expression of *Xbra* in the absence of protein synthesis.

These data suggest a role for the maternal FGFs which is consistent with their expression patterns. There is now good evidence that in the early blastula the FGFs do not act as vegetally localised mesoderm inducing factors but rather they provide a sub-threshold stimulation of the tyrosine kinase signal transduction pathway in the cells of the animal hemisphere. This is further supported by the demonstration that there is low level MAP kinase activity in the animal hemisphere and much of this can be inhibited by overexpression of XFD (LaBonne *et al.*, 1995). This low level of FGF activity in the cells of the animal hemisphere is required for the full repertoire of responses to induction by activin-like molecules, including vg1 (SchulteMerker *et al.*, 1994b), and as such the FGFs can be considered as competence factors necessary for mesoderm induction. The view that the vegetal hemisphere is not a major source of FGF signalling is further supported by recent evidence which demonstrates that, like the cells of the animal hemisphere, vegetal hemisphere cells can express the mesodermal markers *Xbra* and *XmyoD* in response to treatment with FGF. However, unlike animal cells, vegetal cells do not express *Xbra* and *XmyoD* in response to activin treatment. The fact that *Xbra* and *XmyoD* expression is normally excluded from the vegetal hemisphere indicates that an FGF is not a major component of the endogenous vegetal signal (Cornell *et al.*, 1995).

Not all mesodermal genes however, require FGF activity for their initial activation in the mesoderm. These include the other general mesodermal markers *Xsna* and *eFGF* itself. Also the expression of the dorsoanterior markers *goosecoid* and *otx2* are not significantly reduced in XFD embryos. It is significant that the expression of *goosecoid* is unaffected in XFD embryos. *goosecoid* expression does not overlap that of either *eFGF* or *Xbra* and at the start of gastrulation is restricted to the deep layers of the dorsal lip in cells which contribute to the head, which is relatively normal in XFD embryos.

Although it can be seen that there is some regional restriction to the requirement for FGF activity, it is important to note that this requrement is not restricted to the ventral mesoderm. For example, *noggin* which is co-expressed with *Xbra* in the notochord, is somewhat down regulated in XFD injected embryos.

FGF and muscle formation

In the previous chapter we saw that muscle formation is very much reduced in XFD embryos. This is reflected in absence of *XmyoD*, *Xmyf5* and cardiac *actin* expression detectable by *in situ* hybridisation. However, RNAase protection analysis shows that the initial low level expression of *XmyoD* at the start of gastrulation is unaffected in XFD embryos. This indicates that the requirement for FGF activity is for the amplification and maintenance of *XmyoD* , and possibly *Xmyf5*, expression during gastrula stages. This is in keeping with previous results that show the activation of high levels of *XmyoD* by mesoderm inducing factors requires protein synthesis (Harvey, 1991) and indicates that it is likely to be

zygotic FGF expression which is involved in the elaboration of muscle gene expression.

FGFs and anteroposterior gene expression

A wide range of anteroposterior marker genes which are expressed in the mesoderm and ectoderm were examined in XFD embryos. The expression of *otx2*, which is a marker of anterior development in all three germ layers, is relatively unaffected in XFD embryos. This is in keeping with the morphology of XFD embryos and further indicates that the FGFs are not required for the specification of the most anterior body parts up to about the level of the forebrain/midbrain junction. The effects on genes expressed in the midbrain and hind brain are more complex. The expression of the midbrain/hindbrain junction marker *en-2* and the hindbrain markers *krox20* and *HoxB1* is still detected by *in situ* hybridisation in XFD embryos at levels comparable to that seen in uninjected embryos. However, the domains of expression for these genes become spilt around the open blastopore by the abnormal morphogenetic movements. This shows that the mechanism for the establishment of the expression of these genes probably does not involve FGF signalling. The expression these genes is not detected until after the spreading of dorsal tissue around the blastopore is apparent. Therefore it is not possible to say if their domains of expression become split subsequent to their initial specification or whether this pattern reflects a split in the inducing tissue prior to specification.

The activation of expression from *HoxC6* and *A7*, which are normally expressed in the spinal cord and mesoderm of the trunk, is late and at reduced

levels compared to controls. The expression of these genes is not initiated until quite late in gastrulation, therefore it is not clear if the effects on the expression of these *Hox* genes is due to some earlier effect on FGF dependent patterning or whether FGF is directly involved in the regulation of *Hox* gene activity in the gastrula. However, recent data in this lab, obtained using eFGF loaded beads, indicates that the FGFs can activate the expression of the same *Hox* genes, in both the ectoderm and mesoderm of gastrula stage embryos, which suggests that the FGFs are involved in establishing the expression of a subset of *Hox* genes in the trunk and posterior (Pownall *et al.*, submitted). These results are significant because it is now widely accepted that genes of the vertebrate *Hox* clusters are directly involved in the specification of anteroposterior levels along the body axis (McGinnis and Krumlauf, 1992).

Gene expression and the XFD phenotype

The activity of the FGFs is required for the correct regulation of a subset of genes within both the mesoderm and the ectoderm. A number of the FGF sensitive genes such as *Xbra*, *XmyoD* and the *Hox* genes are known to be intimately involved in specification and patterning during vertebrate development. Data in this and the previous chapter show that to a large extent the final phenotype of XFD embryos can be traced back to effects on gene expression that are apparent during gastrula stages. For example, genes which are known to be involved in the specification and patterning of the most anterior structures are relatively little effected in XFD embryos. Whereas the expression of *XmyoD* and some of the

Hox genes is greatly down regulated during gastrula stages and probably accounts for loss of trunk and tail structures in these embryos.

There is now good evidence that the maternal FGFs are required during the initial stages of mesoderm induction. In the blastula stage there is widespread expression of *eFGF*, *FGF-9* and *bFGF* in the animal hemisphere that is necessary for the full range of competence to respond to the vegetal inducers. However, during gastrula and neurula stages *eFGF* and *FGF-3* are expressed in discrete domains in the marginal zone, notochord and posterior. This is very similar to the expression pattern of *Xbra* and suggests that, in addition to a requirement for maternal FGF function to activate the immediate early expression of Xbra, there is a continued requirement for the zygotic activity of the FGFs to maintain *Xbra* expression.

Data in this chapter also suggests a role for the zygotic FGFs in regulating the expression of other genes during gastrula and neurula stages. Unlike *Xbra*, the expression of the myogenic and *Hox* genes is not an immediate early response to mesoderm induction, so it seems unlikely that maternal FGF function has any role in their activation. However, there is considerable overlap between the zygotic expression of the FGFs and the initial expression domains of these genes in the marginal zone and posterior of the embryo. The next chapter provides further support for the view that eFGF is involved in the regulation *Xbra* and *XmyoD* expression within the newly formed mesoderm in the gastrula and neurula.

Chapter 7

eFGF and gene regulation within the mesoderm.

Introduction

The *in vivo* inhibition of FGF function reveals that the activity of the FGFs is required for the normal development of the mesoderm. Recent work in other labs strongly suggests that at least part of the requirement for the FGFs is maternal (Cornell *et al.*, 1995; LaBonne *et al.*, 1995). However, the expression data for *eFGF* makes it likely that there is a continuing requirement for FGF activity within the developing mesoderm in later stages. Data in the last chapter shows that *Xbra* and the myogenic genes are very sensitive FGF inhibition. This chapter examines in more detail the potential role for eFGF in regulating the expression of *Xbra* and *XmyoD* within the nascent mesoderm.

As discussed in Chapter 3, the *Xenopus* homologue of the *Brachyury* gene (*Xbra*) belongs to an increasingly large family of related 'T-box' genes. In all vertebrates examined the expression of *Brachyury* is essentially identical. It is expressed in the nascent endomesoderm at the start of gastrulation. As gastrulation proceeds its expression is rapidly down regulated in all involuted mesoderm apart from the notochord . *Brachyury* expression persists in the tailbud region of vertebrate embryos throughout the period of tail extension. Mutations in the *Brachyury* gene have been identified in the zebrafish and mouse and analysis of the phenotype of these mutants reveals that *Brachyury* function is required for formation of trunk and tail structures and for the differentiation, but not initial specification, of the notochord (Herrmann and Kispert, 1994). The identification of closely related 'T-box' genes expressed in the mesoderm of primitive cephalochordate, urochordates and hemichordates indicates that function of *Brachyury* in the formation of the mesoderm is extremely ancient (Yasuo *et al.*,

1995). The recent identification of a *Brachyury* homologue, which is expressed in the hind gut of *Drosophila*, has further stimulated discussion over the evolutionary origin and possibly endodermal nature of the notochord (Kispert *et al.*, 1994).

The mouse Brachyury protein is a transcription factor which has now been analysed in some detail. The 436 amino acid protein is almost exclusively localised to the nucleus and the nuclear localisation signal has been narrowed down to a region encompassing amino acids 137 to 320. The amino terminal half of *Brachyury* is most highly conserved across species and it is this region that contains the DNA binding 'T-box' domain. A consensus 12-mer recognition site has been identified which can bind *Brachyury* either as a repeat or inverted repeat. Reporter constructs carrying tandem repeats of this sequence have been shown to be activated in the presence of Brachyury protein. Deletion analysis has revealed that in common with many transcription factors Brachyury has a modular organisation and the carboxy terminal contains 2 activation and repression domains. This is in keeping with the reduced conservation of sequence in this region because there are fewer constraints on the primary sequence of an activation domain, where the major requirement is for acidic amino acids, compared to the steric constraints imposed by interaction of a DNA binding domain with specific target sequence (Kispert, 1995).

Inhibition of the FGF signal transduction pathway by the injection of mRNA coding for the dominant negative FGF receptor (XFD) results in embryos in which the trunk and tail are vestigial (Amaya *et al.*, 1991; Isaacs *et al.*, 1994). This phenotype, of trunk and posterior deficiency, failure of notochord differentiation and relatively normal anterior development, bears similarities to that caused by

naturally occurring mutations in the *Brachyury* (T) gene of the mouse (Beddington *et al.*, 1992) and the *no tail* mutation in zebrafish (SchulteMerker *et al.*, 1994c). These are mutations in the direct homologues of the *Xenopus Xbra* gene. There is now considerable evidence linking the activity of the FGFs with the regulation of the expression of the *Xbra* gene. Inhibition of the FGF signal transduction pathway leads to a dramatic reduction of *Xbra* expression through gastrula stages (Amaya *et al.*, 1991; Isaacs *et al.*, 1994). The expression pattern of *eFGF* in the periblastoporal region, notochord and later in the tailbud is also strikingly similar to that of *Xbra* (Isaacs *et al.*, 1995; Smith *et al.*, 1991). It is tempting to speculate that the underlying mechanism involved in the formation of the phenotype of *Brachyury* mutants and FGF dominant negative embryos is similar.

The development of muscle lineages is greatly reduced in XFD injected embryos with a concomitant reduction in muscle gene expression, including the basic helix-loop-helix (bHLH) transcription factors *XmyoD* and *Xmyf5* (Amaya *et al.*, 1991, Chapter 6). These factors have been shown to be capable of acting as dominant activators of muscle specific gene expression in number of systems including *Xenopus* (Weintraub, 1993; Rudnicki and Jaenisch, 1995). The demonstration of the complete lack of skeletal muscle development in mice carrying double null mutations for *XmyoD* and *Xmyf5* further emphasises the vital role that they have in the development of the muscle lineages (Rudnicki *et al.*, 1993).

This chapter contains experiments which further elucidate the role of eFGF in regulating mesodermal gene expression. Data show that cell-cell signalling is required to maintain the expression of *Xbra* and *XmyoD* in the cells of the

periblastoporal region during gastrula stages and that eFGF can replace that requirement in a novel cell culture system. Further complexities to the relationship between eFGF and Xbra are indicated by data showing that they can each activate the expression of the other. This suggests that an autoregulatory loop of Xbra and eFGF is involved in the development of the mesoderm.

Materials and methods

Embryological methods

Embryo production was as per general methods. Animal caps explants taken as per general methods. The specific activity of the mesoderm inducing factors was determined by the serial dilution assay (Godsave and Slack, 1988). Recombinant *Xenopus* bFGF was prepared as per Kimelman *et al.*, (1988). Recombinant *Xenopus* eFGF was prepared as per Chapter 4. Recombinant bovine activin A was a gift from Innogenetics (Belgium). Disaggregated marginal zone cell cultures were prepared by dissecting gastrula stage 10.5 to stage 11 embryos into either dorsal and ventral 1/2s or dorsal 1/4s and lateroventral 3/4s. Animal hemisphere tissue was then removed down to the floor of the blastocoel and as much as possible of the vegetal core material dissected away. Disaggregation and factor treatment was as per general methods.

RNA injections

RNA injections were carried out as per general methods. The dominant negative FGF receptor (XFD) and control receptor (d50) constructs are those used by Amaya *et al.*, (1991). The pSP64-ß-globin plasmid is that of Krieg and Melton,

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The Fibroblast Growth Factor Family in the Early Development of *Xenopus laevis*.

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(1984). The pSP64-XbFGF plasmid is as used by Thompson and Slack, (1992). The pSP64-eFGF plasmid is as used by Isaacs *et al.*, (1994). The pSP64-Xbra is as used by Cunliffe and Smith, (1992).

RNAase protections

As per general methods

Results

Inhibition of eFGF activity by the dominant negative receptor (XFD)

The main focus of this thesis is the role and activities of eFGF during early *Xenopus* development. It is therefore important to show that XFD is able to inhibit the activity of eFGF. Table 1 demonstrates the usefulness of this reagent as an inhibitor of eFGF function. Coinjection of *eFGF* with the dominant negative FGF receptor leads to a marked reduction in the autoinducing activity of *eFGF* mRNA. This means that inhibition of eFGF activity will in part be responsible for the effects seen in XFD injected embryos. This experiment raises the issue of the specificity of the block on FGF signalling produced from the dominant negative FGF receptor. The dominant negative approach works because of the ligand dependant dimerisation of the mutant receptor with wild-type receptor (Ueno *et al.*, 1992). This implies that signalling will be blocked from any ligand that can bind to a receptor complex which contains the dominant negative receptor. All members of the FGF ligand family have been shown to bind to at least one isoform of FGF-R1, FGF-R2 or FGF-R3 (Johnson and Williams, 1993). Furthermore, it has been shown that a similarly truncated mouse FGF-R1 will act as a dominant inhibitor of

signalling from FGF-R1, FGF-R2 and FGF-R3, presumably through the formation of FGF-R heterodimers (Ueno *et al.*, 1992). However, the dominant negative FGF receptor is the R1 IIIc isoform and FGF-3, KGF, FGF-8 will not bind to homodimers of R3 IIIc. The problem is that it is not known if these ligands will bind to heterodimers containing the R1 IIIc isoform. The story is complex and it is likely that the inhibitory activity of XFD should be tested empirically for each ligand. On this basis it can be said with confidence that XFD blocks the activity of bFGF, eFGF and FGF-9 (Amaya *et al.*, 1991; Isaacs *et al.*, 1994; Song and Slack, 1996).

Table 1 Inhibition of mesoderm induction in animal caps by injection of dominant negative FGF receptor (XFD) mRNA.

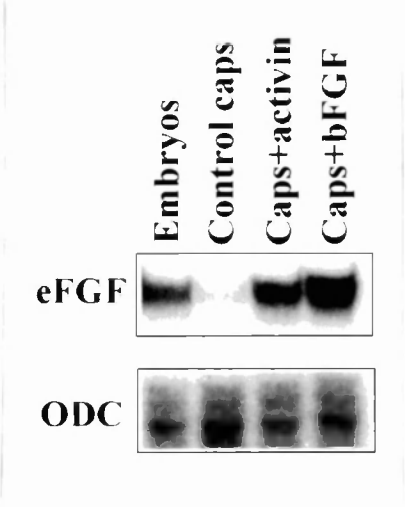
Injection	Uninduced	Induced	*n*
Water	10	0	10
20 pg eFGF mRNA	0	10	10
+4 ng d50 mRNA	0	7	7
+4 ng XFD mRNA	8	2	10

RNA was injected into both blastomeres of 2-cell stage embryos. d50 is a non-functional receptor control. XFD is the dominant negative FGF receptor. Animal caps were removed at stage 9 and were cultured for 3 days. At this stage the presence of fluid-filled vesicles indicates the formation of mesoderm.

The induction of eFGF expression by FGF, activin and Xbra

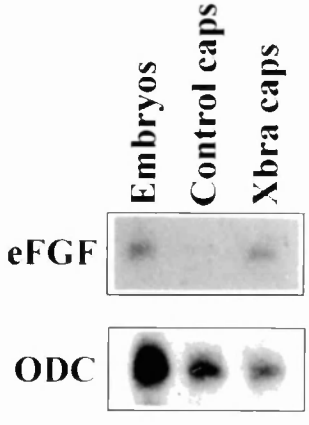
Although *eFGF* is expressed at low levels maternally, it is most highly expressed in the periblastoporal region during gastrula stages (Isaacs *et al.*, 1992; Isaacs *et al.*, 1995). This suggests that its zygotic expression might be activated by mesoderm inducing factors. Fig. 1 is the result of an RNAase protection assay that shows treatment of animal cap explants with the dorsal-type inducer activin and the ventral-type inducer bFGF leads to the activation of *eFGF* expression.

Fig. 1. RNAase protection analysis of *eFGF* expression in animal caps by treatment with mesoderm inducing factors.



Animal cap explants were taken at stage 8 and cultured until stage 11. 6 µg of total RNA from untreated control caps, activin treated caps(10u/ml) and bFGF treated caps (10 u/ml) were analysed by RNAase protection for *eFGF* and *ODC* expression.

Fig. 2. RNAase protection analysis of *eFGF* expression in animal caps following injection of *Xbra* mRNA.



Animal caps were taken from water injected control embryos and embryos injected with 4 ng of *Xbra* mRNA. 5 µg of total RNA from caps at stage 11 were analysed by RNAase protection for *eFGF* and *ODC* expression.

Thus the zygotic expression of *eFGF* is a property of both dorsal and ventral type mesoderm.

Cunliffe and Smith, (1992) have demonstrated that the injection of mRNA coding for *Xbra* leads to autoinduction of ventral-type mesoderm in animal caps. The mesoderm that is induced is very similar in character to that induced by the treatment of animal caps with members of the FGF family. This raises the possibility that Xbra will also be able to induce the zygotic expression *eFGF*. RNAase protection analysis of animal caps taken from embryos that have been injected with *Xbra* mRNA shows that this is indeed the case (Fig. 2). In common with other mesoderm inducing factors (Smith *et al.*, 1991), eFGF is able to strongly induce the expression of *Xbra* in animal caps (see Fig. 4, Chapter 4). As has already been discussed, zygotic expression both *eFGF* and *Xbra* is activated in very similar domains within the nascent mesoderm of the marginal zone. Therefore, it is possible that this cross activation of each others expression may actually occur during normal development and that a positive feedback loop of this kind may be involved in the elaboration the expression of both *Xbra* and *eFGF*. Further support for this is provided by gene expression data from dominant negative FGF receptor injected embryos that show that *Xbra* expression is greatly reduced from the start of gastrulation (Amaya *et al.*, 1993; Isaacs *et al.*, 1994). Interestingly, the initial expression of *eFGF* at the start of gastrulation is not down regulated in XFD embryos even at a time when *Xbra* expression is much reduced. However, as gastrulation proceeds the level of *eFGF* expression falls in XFD embryos (Isaacs *et al.*, 1994). This indicates that the activation of *Xbra* expression requires a functional FGF signal transduction pathway, whereas the

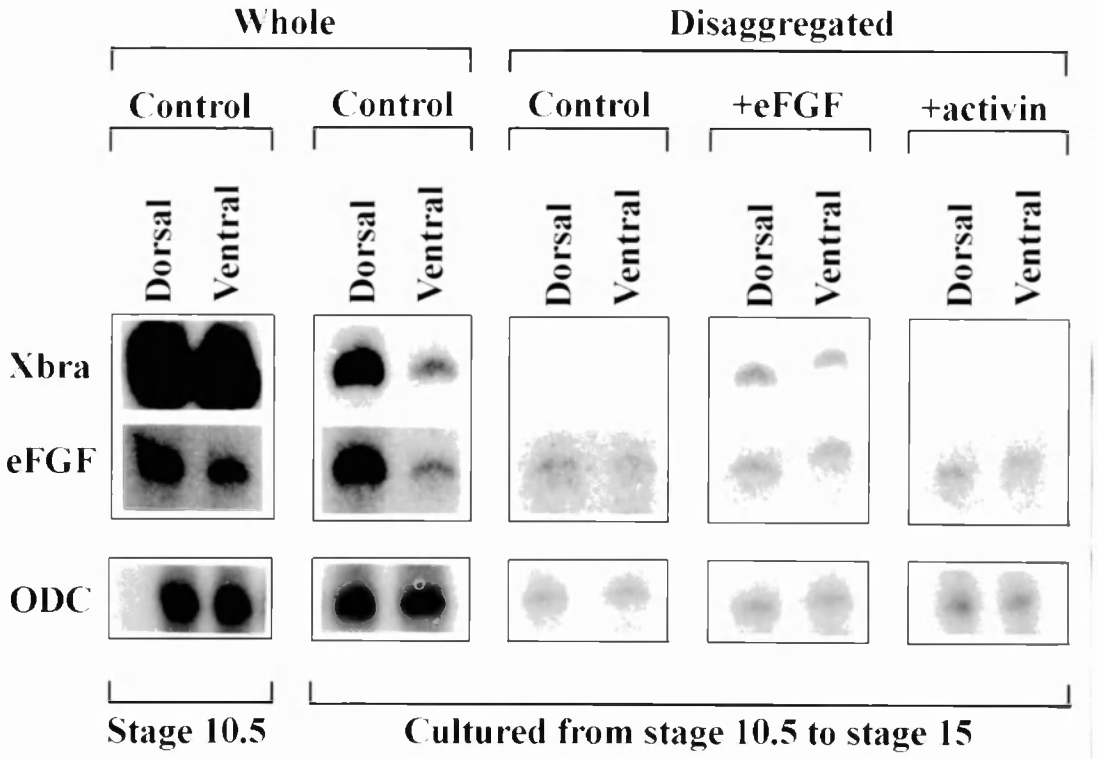
activation of eFGF expression does not and that it is only during gastrula stages that *Xbra* is required to maintain eFGF expression. Furthermore, it seems likely that not only does the initial activation of *Xbra* expression require a functional FGF signal transduction pathway but there is also a continued requirement for FGF activity to maintain *Xbra* expression during gastrula stages.

Xbra expression in disaggregated marginal zone explants

The notion that eFGF continues to be required for the expression of *Xbra* in the mesoderm of the blastopore during gastrula and neurula stages has been directly tested. The approach was to desegregate the cells of blastopore region explants from gastrula stage 10.5 embryos and culture in the presence of various factors until the mid-neurula stage 15. At this stage the cells were harvested and analysed for the expression of *Xbra* and *eFGF*. The object of the cell disaggregation protocol is to remove any autocrine secreted factors from the environment of the cells by massive dilution into the bulk medium.

Fig. 3 shows that the dorsal and ventral blastopore pieces from stage 10.5 express *Xbra* and *eFGF*. These same pieces, when cultured intact until stage 15, continue to express all three genes. However, when the explants are disaggregated before culture, the expression of *Xbra* is completely extinguished both in the dorsal and ventral derived cells. If however eFGF protein is included in the medium, *Xbra* continues to be expressed in both dorsal and ventral derived cell cultures. eFGF maintains *Xbra* expression at levels comparable to the ventral control pieces but at a somewhat lower level than the dorsal control. Activin also has *Xbra* maintenance activity but, with the concentration of factors used in this

Fig. 3. RNAase protection analysis of *eFGF* and *Xbra* expression in disaggregated gastrula dorsal and ventral marginal zone explants.



3 μg of total RNA from each of the cultures were analysed by RNAase protection for the expression of *Xbra*, *eFGF* and *ODC* . Whole dorsal and ventral explant controls were analysed at gastrula stage 10.5 and after culturing to neurula stage 15. Disaggregated untreated control cells, and eFGF treated (4 u/ml), and activin treated (16 u/ml) cells were analysed after culturing from stage 10.5 to stage 15.

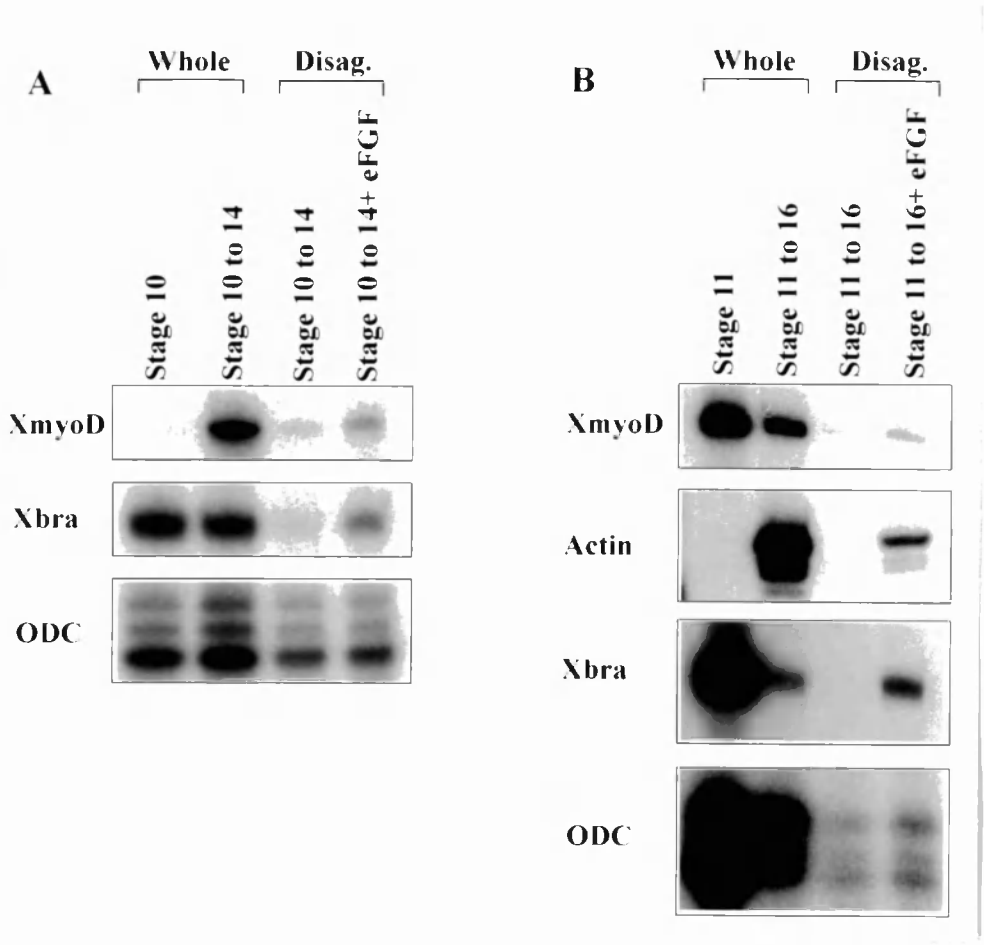
experiment (eFGF at 4 u/ml and activin at 16 u/ml), is less effective than eFGF. Note, that although the total amounts of RNA analysed in each lane is the same, the expression of the ornithine decarboxylase (ODC) loading control is significantly down regulated in disaggregated cell culture samples. ODC is an enzyme that is involved in the synthesis of polyamines and is ubiquitously expressed in *Xenopus* embryos and explants. However, in tissue culture the ODC has been shown to be highly regulated at the level of transcription and translation in response to a large number of stimuli including hypotonic shock and even substrate independent growth (Morrison and Seidel, 1995; Wallon *et al.*, 1995). The down regulation of ODC expression may simply reflect the altered metabolic state of cells under disaggregated culture conditions. However, all disaggregated cultures are similarly affected and therefore this phenomenon does not compromise within group comparisons.

It can be concluded that eFGF is able to maintain *Xbra* expression in cells of dorsal and ventral blastopore regions during gastrula and neurula stages. This maintenance function is distinct from primary mesoderm induction and constitutes a novel activity for eFGF during gastrulation. This result has also been reported by (Schulte-Merker and Smith, 1995)

eFGF and XmyoD expression

Expression of the myogenic basic helix-loop-helix transcription factor *XmyoD* is also greatly down regulated in XFD injected embryos as is the subsequent expression of the muscle differentiation marker *actin*. There is a considerable body of literature that indicates intercelluar signalling is required during gastrula

Fig. 4. RNAase protection analysis of myogenic gene expression in disaggregated gastrula lateroventral marginal zone explants.



5 µg of total RNA from each of the cultures were analysed by RNAase protection for the expression of *XmyoD*, *cardiac actin*, *Xbra* and *ODC*.

A: Whole ventrolateral marginal zone explant controls were analysed at gastrula stage 10 and after culturing to neurula stage 14. Untreated disaggregated control cells and eFGF treated (10 units/ml) disaggregated cells were analysed after culturing from stage 10 to stage 14.

B: Whole ventrolateral marginal zone explant controls were analysed at gastrula stage 11 and after culturing to mid neurula stage 16. Untreated disaggregated control cells and eFGF treated (10 units/ml) disaggregated cells were analysed after culturing from stage 11 to stage 16.

stages for the subsequent differentiation of muscle lineages (Gurdon, 1988; Gurdon *et al.*, 1992; Gurdon *et al.*, 1993). During gastrula stages *XmyoD* is expressed in the periblastoporal region, although unlike *eFGF* and *Xbra* its expression is excluded from the region of the organiser. These data raise the possibility that eFGF may be involved in the amplification of the initial low level *XmyoD* expression at the start of gastrulation and its maintenance during the development of the muscle lineages. This has been tested using a similar disaggregation protocol to that described above.

At the start of gastrulation there is only a very low level *XmyoD* expression but levels greatly increases as gastrulation proceeds (see Fig. 1, Chapter 5). In order to test if eFGF is also able to activate *XmyoD* expression in disaggregated cell culture the lateroventral 3/4 of the marginal zone was explanted at stage 10 and then cultured either intact or disaggregated (plus or minus eFGF) until neurula stage 14 and then assayed for the expression of *XmyoD* (*Xbra* has been included as a positive control). Fig. 4A shows that there is only a very low level of *XmyoD* expression in early gastrula explants. If these explants are cultured intact until neurula stage 14 there is a considerable increase in *XmyoD* expression. On the other hand, in disaggregated cell culture there is very little increase in the expression of *XmyoD* between stage 10 and 14. However, the inclusion of eFGF in the medium of the disaggregated cell cultures results in a significant increase in *XmyoD* expression.

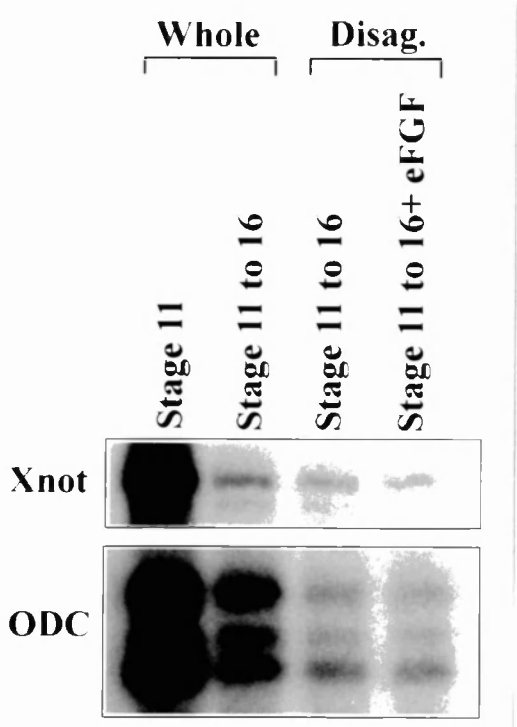
In order to test if eFGF is also able to maintain *XmyoD* expression and activate the expression of muscle differentiation markers in disaggregated cell culture the periblastoporal region from gastrula stage 11 embryos was dissected

into dorsal 1/4s and lateroventral 3/4s and then cultured either intact or disaggregated (plus or minus eFGF) until neurula stage 16. The ventrolateral cultures were then assayed for the expression of *XmyoD*, cardiac *actin* and *Xbra*. Figure 4B shows that at stage 11 the lateroventral region is strongly expressing *XmyoD*. If this explant is cultured intact until stage 16 the *XmyoD* expression has increased considerably and has begun to express muscle *actin*, indicating that at least some muscle differentiation is taking place in the explant. Again it can be seen that that the expression of *XmyoD* is greatly down regulated following disaggregation and there is no cardiac *actin* expression. This confirms previous findings that myogenic precursors do not become committed to form muscle in isolation until late gastrula stages. If however eFGF protein is included in the culture medium the level of *XmyoD* expression is elevated and significantly the cells also express cardiac *actin*. The ability of eFGF to rescue the expression of *XmyoD* in disaggregated cell culture is perhaps less dramatic than is the case for the rescue of *Xbra* expression. However, these preliminary results, in a particular the ability of eFGF to allow the expression of cardiac *actin* in disaggregated cell culture indicate that the activity of the FGFs might be involved in the amplification and maintenance of *XmyoD* expression in normal development and that eFGF in particular may in part mediate the 'community effect' for muscle differentiation (Gurdon, 1988).

eFGF and Xnot expression

The dorsal quarters from the above experiment were assayed for the expression of *Xnot*, which is a homeobox gene isolated independently by two groups (Gont

Fig. 5 RNAase protection analysis of *Xnot* expression in disaggregated gastrula dorsal marginal zone explants.



5 µg of total RNA from each of the cultures were analysed by RNAase protection for the expression of *Xnot* and *ODC*. Whole dorsal marginal zone explant controls were analysed at gastrula stage 11 and after culturing to mid neurula stage 16. Untreated disaggregated control cells and eFGF treated (10 units/ml) disaggregated cells were analysed after culturing from stage 11 to stage 16.

and De Robertis, 1993; Von Dassow *et al.*, 1993a). *Xnot* is expressed in the periblastoporal region and developing notochord during gastrula stages and has been shown to be down regulated at the start of gastrulation in XFD injected embryos (Von Dassow *et al.*, 1993a). The result obtained with *Xnot* expression in disaggregated dorsal explants was somewhat unexpected (Fig. 5). *Xnot* expression is not down regulated in disaggregated cell culture and is not up regulated by the inclusion of eFGF in the culture medium. This indicates that by stage 11 the expression of *Xnot* in the dorsal mesoderm has become cell autonomous and not sensitive to FGF signalling. This is in contrast to the activation of *Xnot* expression within the organiser, which is dependent upon FGF signalling (Von Dassow *et al.*, 1993b).

Attempted rescue of the dominant negative FGF receptor phenotype

Much of the XFD phenotype in *Xenopus* can be accounted for by a sensitive effect on dorsal cell movements during gastrulation that leads to the failure of the blastopore to close. It is possible that the underlying cause of the XFD and Brachyury mutant phenotypes is the same, and that this is the lack of Brachyury function. A naive view suggests that it might be possible to rescue the XFD phenotype by simply returning Brachyury function. This has been attempted by coinjecting *Xbra* and XFD mRNA at the pigment boundary of all blastomeres or just the 2 dorsal blastomeres in the 4-cell embryo.

In order to sensitise this assay a preliminary study was carried out to find a threshold concentration of XFD that would just produce the open blastopore phenotype. This value was found to be 50 pg/ dorsal blastomere at the 4-cell

stage. A further complication is that overexpression of *Xbra* also leads to a phenotype of arrested gastrulation, due to the formation of ectopic mesoderm in the animal hemisphere (Cunliffe and Smith, 1992). Such embryos characteristically have animal hemispheres with a wrinkled thickened appearance. The amount of *Xbra* mRNA injected (200-400 pg/blastomere at 4-cell stage) was an amount that just caused failure of the blastopore to close when injected on its own. The results of this experiment are shown in Table 2. Coinjection of the wild-type receptor was included as a control for rescue. It can be seen that a 2-fold excess of wild-type receptor mRNA over that of XFD mRNA is sufficient to completely rescue the open blastopore phenotype in most embryos. By contrast coinjection of *Xbra* mRNA does not show any signs of rescuing closure of the blastopore.

Table 2 Attempted rescue of the XFD phenotype by Xbra and the wild-type FGF receptor (XFR)

Injection	Open blastopore	Closed blastopore	Other	*n*
Xbra mRNA				
4x 400 pg	12x thickened animal cap	0		12
XFD mRNA				
4x 50 pg	42x typical XFD phenotype	1x normal	3x sick	46
2x 50 pg dorsal	8x typical XFD phenotype	3x normal		11
XFD+XFR mRNA				
4x 50 pg+100 pg	4x XFD phenotype	32x normal	4x sick	40
XFD+Xbra mRNA				
4x 50 pg+400pg	20x some thickening of animal cap	0	2x sick	22
2x 50 pg+200 pg dorsal	12x XFD phenotype	0	1x sick	13

Embryos were injected with specified amounts of mRNA at the 4-cell stage. They were scored for blastopore closure between late gastrula stage 13 and neurula stage 16.

This is clearly a technically difficult experiment with a great many variables. An experiment of this sort with a negative results cannot be conclusive but it does provide some evidence that the open blastopore phenotype is not due only to an interference with the regulation of *Xbra* expression.

Discussion

Mesoderm formation

Data obtained using the dominant negative activin receptor suggest that this signalling pathway is required for the expression of all mesodermal genes (Cornell *et al.*, 1995). Hence the blocking of the activin signalling pathway in the embryo results in a complete absence of all mesodermal structures (Hemmati-Brivanlou *et al.*, 1992). This is in contrast to data obtained using the dominant negative FGF receptor which indicates that FGF function is only required for the correct regulation of a subset of genes that are expressed in the newly formed mesoderm (Cornell and Kimelman, 1994; Isaacs *et al.*, 1994; LaBonne and Whitman, 1994). It is likely that the FGFs have multiple and possibly independent roles in regulating gene expression within the nascent mesoderm. However, at present the best characterised role of FGF is in regulating the expression of the gene *Xbra.*

FGF and the regulation of Xbra expression

Xbra is the *Xenopus* homologue of the transcription factor *Brachyury* which has been shown to have an important role in the formation of the mesoderm in a number of vertebrates (reviewed by Herrmann and Kispert, 1994). Data indicate that *Xbra* plays a similar role in the development of *Xenopus*. *Xbra* expression is

activated in response to mesoderm induction by activin and the FGFs (Smith *et al.*, 1991), including eFGF (Isaacs *et al.*, 1994). Furthermore, overexpression of *Xbra* in animal caps leads to the activation of a number of mesodermal marker genes and induces the formation of ventral-type mesoderm (Cunliffe and Smith, 1992). Just as with FGF the response to mesoderm induction by Xbra can be modified to a more dorsal type. For example the coinjection of Xbra mRNA with *noggin* or *Pintallavis* mRNA results in the formation of notochord (Cunliffe and Smith, 1994; OReilly *et al.*, 1995). Interestingly the induction of mesoderm and *Xbra* expression in animal caps in response to injection of *Brachyury* mRNA requires a functional FGF signal transduction pathway (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; Schulte-Merker and Smith, 1995). The explanation for this may well rest with the observation that *Xbra* activates the expression of eFGF and that subsequent FGF signalling is required for the full development and maintenance of the mesodermal state (Isaacs *et al.*, 1994). The ability of Xbra and eFGF to activate the expression of each other suggests that they may be components of an autocatalytic loop involved in the formation of the mesoderm in the late blastula.

At the start of gastrulation *eFGF* and *Xbra* are expressed in the mesoderm of the blastopore region. When explants are taken from this region, and the cells are dissociated in divalent cation free medium, the expression of *Xbra* is rapidly down regulated. This indicates a requirement for cell-cell signalling in the maintenance of *Xbra* expression in the blastopore region. Experiments in this thesis and elsewhere show that the addition of eFGF and bFGF to the culture medium maintains the expression of *Xbra* in these dissociated cell cultures

(Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). Given their coexpression in the blastopore region, it is likely that eFGF contributes to the *Xbra* maintenance activity in the embryo and that interference with this function in the gastrula underlies at least some aspects of the dominant negative FGF receptor phenotype. The notion that eFGF is involved in maintaining Xbra expression during gastrula stages further supported by the demonstration that ectopic expression of *Xbra* results from injection of a plasmid which drives eFGF expression after the MBT (Isaacs *et al.*, 1994). Data in this thesis show that *eFGF* is also coexpressed with *Xbra* in the notochord in the late gastrula and neurula stages, suggesting that eFGF may also be involved in the regulation of *Xbra* expression in the dorsal midline .

Brachyury mutants in other organisms

A number of naturally occurring mutations in the homologues of the *Xbra* gene have been identified in other organisms. The phenotypes of *T* mutant mice and *no tail* mutant zebrafish exhibit a deficiency in trunk and posterior structures (reviewed by Herrmann, 1995; Herrmann and Kispert, 1994). This has similarities to the phenotype of *Xenopus* embryos in which the activity of the FGFs has been inhibited. At present the extact role of *Brachyury* in mesoderm formation is unclear. Certainly its function is not required for the formation of the mesoderm *per se* because in mouse and zebrafish *Brachyury* mutants the development of anterior mesodermal structures, including anterior somites is relatively normal. Furthermore, Brachyury is not required for the initial specification of the notochord in zebrafish *no tail* mutants because the dorsal midline cells still retain the ability to

induce floor plate in the overlying neural keel. However, *no tail* function is required for the terminal differentiation of the notochord (reviewed Schulte-Merker, (1995)). It is likely that the absence of differentiated notochord in *Xenopus* embryos injected with the dominant negative FGF receptor is due to an interference with the regulation of *Xbra* function in the dorsal midline.

FGF, brachyury and cell movements

It has been suggested that in *T* mutant mice abnormal cell movements within the primitive streak lead to the characteristic posterior truncation (Beddington *et al.*, 1992). Gastrulation movements are also disturbed in *Xenopus* embryos injected dorsally with the dominant negative FGF receptor. Instead of involuting and extending along the anteroposterior axis, as in normal development, the dorsal mesoderm spreads laterally and ventrally around the open blastopore (Isaacs *et al.*, 1994). Given the close link between FGF and Xbra activity and the intimate relationship between morphogenesis and patterning during gastrulation, it is tempting to suggest that the reduction of posterior structures in both *Brachyury* mutant organisms and FGF dominant negative receptor embryos results primarily from abnormal cell movements in the dorsal mesoderm. Certainly there is evidence from studies in the mouse which indicates that the extracellular matrix of *Brachyury* mutant derived cells is very much reduced in quantity from that of wild-type cells (Hashimoto *et al.*, 1987). However, the coinjection of *Xbra* mRNA fails to rescue the open blastopore phenotype of XFD injected embryos suggesting that the aberrant cell movements caused by inhibition of FGF activity is not simply due to an interference with *Xbra* function.

Clearly there are many other potential down stream targets of the FGF signal transduction pathway, such as matrix molecules and their receptors, that might be account for the aberrant cell movements seen in XFD injected embryos. However, it is possible that the alteration in cell movements seen in XFD injected embryos are due to a direct effect on cell behaviour that does not necessarily involve regulation of gene activity at the level of the nucleus. It has been demonstrated that signalling from polypeptide growth factors such as the FGFs can have profound effects on the organisation of the cytoskeleton and motility of cells acting through the ras related small GTPases rho and rac (Nobes and Hall, 1995). Given the fact that ras has been shown to play a pivotal role in transducing the mesoderm inducing activity of the FGFs, it is conceivable that signal transduction through rho and rac is involved in regulating cell motility during gastrulation. Interestingly, targeted disruption of the FGF-R1 in mouse leads to abnormality of cell movement through the primitive streak without a concomitant reduction in the expression of *Brachyury*.

In the mammals FGFs have been shown to be capable of stimulating the migration of endothelial cells during angiogenesis (reviewed Basilico and Moscatelli, (1993)). FGF activity has also been shown to be important in controlling cell movement in *Drosophila* and *C.elegans*. In *Drosophila* a mutation in the *breathless* gene, which is an FGF receptor homologue, inhibits migration of tracheal cells and a population of cells in ovary (Murphy *et al.*, 1995; Reichmanfried *et al.*, 1994 ; Reichmanfried and Shilo, 1995). In *C.elegans* the *egl-15* gene also codes for an FGF receptor which is involved in directing migration of sex myoblasts during development (DeVore *et al.*, 1994).

The autoregulatory loop

The evidence for an autoregulatory loop involving eFGF and Xbra is quite strong. However, there are still many unanswered questions as to the nature of this loop and at what stage in development does the regulatory loop become important. Does the available data from *Brachyury* mutant organisms support the existence of such a loop? Examination of gene expression in mouse and zebrafish *Brachyury* mutants can help cast light on these matters.

In both mouse and zebrafish, *Brachyury* alleles have been identified which are transcribed but when translated are presumed to produce non-functional proteins. The examination of *no tail* and *T* mutants shows that the initial expression of *Brachyury* is present in the germ ring and primitive streak (Herrmann, 1991; SchulteMerker *et al.*, 1994a); although in the zebrafish the germ ring expression is somewhat reduced. This is in contrast to the expression of *Brachyury* in XFD injected *Xenopus* embryos where *Xbra* expression is almost completely absent. However, in both zebrafish and mouse no expression of *Brachyury* is found in cells of the presumptive notochord.

Unfortunately we do not know the expression of *eFGF* homologues in *Brachyury* mutants. However, the data in Chapter 4 shows that at the start of gastrulation in XFD *Xenopus* embryos, even though *Xbra* expression is almost absent, there is very little effect on the initial expression of *eFGF*. But later in gastrulation the level of *eFGF* expression is reduced in XFD embryos suggesting that *Xbra* is required for the maintenance of some element of *eFGF* expression at this stage. The requirement for Xbra to maintain *eFGF* expression is never as complete as is the reverse case because eFGF expression is never totally lost

from XFD embryos or in disaggregated cell culture where *Xbra* expression is absent.

Clearly this is still an incomplete data set but it is possible to draw some conclusions. The issue over the initial activation of zygotic *Xbra* and *eFGF* is somewhat contentious. As has been previously discussed, there is a considerable body of data indicating that there is a requirement for maternal FGF signalling to allow the activation of *Xbra* expression by the presumed activin-like vegetal inducer. However, it has been argued that the requirement for FGF signalling is purely zygotic and that even in the absence of a functional FGF signal transduction pathway activin can induce a transient 'spike' of *Xbra* expression which then in turn activates the zygotic expression of *eFGF* (Schulte-Merker and Smith, 1995).

If we just focus on the issue of maintenance, it is clear that within the nascent mesoderm of the embryo there is an absolute requirement for FGF function to maintain expression of *Brachyury*. It is only in later gastrula stages that *Brachyury* contributes to the maintenance of *eFGF* expression and it is during these stages that that a positive feedback regulatory loop exists between *eFGF* and *Brachyury*. In the absence of functional Brachyury protein there is still persistent *Brachyury* mRNA expression in the germ ring of *no tail* mutants. However, this level of expression is lower than in wild-type embryos and argues that in this region of the embryo the feedback loop functions to maintain and possibly amplify the initial level of *Brachyury* expression activated by the maternal inducers. The complete absence of *Brachyury* expression in the notochord of *T* and *no tail* mutants argues that the terminal differentiation of and the normal

expression of *Brachyury* within the notochord is completely dependent upon the feedback loop. The chordoneural hinge of the tailbud is related by lineage to the late dorsal lip so it seems likely that the expression of Brachyury in this region is also dependant upon the feedback loop.

This model allows a number of predictions to be made about gene expression in a hypothetical *Xenopus* mutant in which a non-functional *Xbra* protein is produced. At the start of gastrulation both *eFGF* and *Xbra* mRNA will be expressed in the circumblastoporal region although *Xbra* expression will be at a somewhat lower level than normal. During later gastrulation the levels of both *eFGF* and *Xbra* will persist but at reduced levels around the lateral and ventral aspects of the closing blastopore. *Xbra* and *eFGF* transcripts will be absent from presumptive notochord cells of the dorsal midline and chordoneural hinge of the developing tailbud.

FGF and the development of muscle lineages

Although the phenotype of embryos lacking a functional FGF signalling pathway is similar to *Brachyury* mutant embryos there are differences and these differences serve to highlight additional processes for which FGF function is required. Most notably a number of anterior somites are present in both mouse and zebrafish *Brachyury* mutants whereas there is a complete absence of somites in *Xenopus* embryos in which FGF function is inhibited. This indicates that the FGFs are required for the development of the somites and in particular the muscle lineages in *Xenopus* and that this function is independent of the FGF role in regulating *Brachyury* expression.

It has previously been shown that cell-cell signalling is required for the expression of muscle cell specific gene expression (Gurdon, 1988; Gurdon *et al.*, 1992). Data in this chapter confirm this by showing that in disaggregated cell culture the expression of *XmyoD* is greatly reduced and muscle *actin* fails to be expressed. However, the inclusion of eFGF in the culture medium considerably rescues *XmyoD* expression and even allows the expression of some muscle *actin*. This work is still at an early stage so it is not yet possible to say if the inclusion of eFGF is sufficient to allow full muscle differentiation but it is at least suggestive that eFGF may contribute to the 'community effect' in muscle differentiation proposed by Gurdon and co-workers.

eFGF and *Xbra* are expressed in the same tissues during gastrula and neurula stages and therefore eFGF can be considered as an autocrine signal in regulating *Xbra* expression. During gastrulation, unlike *eFGF*, *XmyoD* expression is strongest in cells which have already involuted, and is excluded from the dorsal midline (Hopwood *et al.*, 1992). This suggests that eFGF provides a paracrine signal involved in muscle development, initially from an adjacent cell layer in the circumblastoporal region and possibly later from the notochord. This is in keeping with recent work in avians suggesting that signals from the dorsal midline are required for the stabilisation and maintenance of the myogenic lineages (Pownall *et al.*, 1996), given that *eFGF* is also expressed in the notochord it is possible that it may contribute to this midline signal.

Again there are suggestions from *Drosophila* that the association of FGF with the development of muscle lineages is ancient. The expression of a second *Drosophila* FGF-receptor homologue (*DFR2*) is restricted to the mesoderm of the

ventral furrow. The expression of *DmyoD* (*nautilus*) and muscle fibre formation is greatly reduced in embryos carrying a deletion which spans *DFR2* (Shishido *et al.*, 1993).

In conclusion there is now good evidence to suggest that the FGFs play an important role in regulating mesodermal gene expression, both during the period of primary mesoderm induction in the blastula, and subsequently in gastrula and neurula stages of *Xenopus laevis*.

Chapter 8

Overview: Perspectives on the role of the FGFs in early development.

The objective of this chapter is to integrate the experimental evidence presented in this thesis with data that has not already been directly discussed. A model for the role of the fibroblast growth factor family in the early development of *Xenopus* is presented. This is discussed in the context of data obtained form mutational analysis of FGF signalling components in the mouse.

The FGFs and anteroposterior development

The inhibition of the FGF signal transduction pathway *in vivo* has provided much information concerning the role of these factors in early development. Another approach that has proved useful in the analysis of FGF function has been the overexpression of the FGF ligands in the embryo. Injection of synthetic mRNA coding for secreted FGFs, such as eFGF, into the zygote or early cleavage stage embryo demonstrates the potent mesoderm inducing activity of these factors (Isaacs *et al.*, 1994; Thompson and Slack, 1992). However, the formation of large quantities of ectopic mesoderm in the animal hemisphere of such embryos blocks normal gastrulation movements, making this an unsatisfactory approach for the analysis of FGF function during later development. These problems associated with mRNA injection can be overcome by the use of DNA constructs that drive expression after the MBT. Several studies show that large quantities of mRNA transcribed from injected DNA plasmids of this type do not accumulate until the late blastula or early gastrula stage, by which time the competence of the animal hemisphere to respond to mesoderm inducing factors is fading (Christian *et al.*, 1992; Smith *et al.*, 1993). When such a construct, which drives *eFGF* expression under the control of a cytoskeletal actin promoter, is injected into the two cell

embryo the majority of embryos gastrulate normally. During later development, however, these embryos exhibit reductions in the development of anterior structures and a gross enlargement of the posterior, particularly the proctodeum. A similar phenotype has been reported when *eFGF* mRNA is injected into zebrafish embryos (Griffin *et al.*, 1995).

The anterior reductions and over development of posterior structures caused by the late overexpression of *eFGF* is in many ways the converse of the dominant negative FGF receptor phenotype and suggests that the FGF system has an important role in establishing the anteroposterior (A-P) pattern of the embryo during gastrula and later stages. There is a body of embryological data that suggests that during A-P specification of the nervous system the default state is anterior and that the acquirement of posterior fate requires the action of a putative "posteriorising" influence (reviewed by Slack and Tannahill, 1992). The presence of FGFs in the posterior of the embryo and the posteriorised phenotype of embryos overexpressing *eFGF* make the FGFs good candidates for such a posteriorising agent.

There is now a considerable amount of data that support this view. Cox and Hemmati-Brivanlou, (1995) have recently showed that posterior axial tissue can induce the expression of midbrain and hindbrain markers in prospective forebrain tissue, confirming the idea of a dominant posteriorising influence. They further showed that this influence can be mimicked by bFGF. However, the non-secreted nature of bFGF and its rather widespread expression pattern during gastrula stage make it an unlikely candidate to fulfil this role. Clearly based upon expression data alone eFGF and FGF-3 are the best candidates amongst the known

Xenopus FGFs to be involved in this process. Pownall *et al.*, (submitted) have shown that eFGF supplied on heparin beads can activate the expression of a subset of posterior *Hox* genes, including *HoxC6*, in both the neuroectoderm and mesoderm during gastrula stages. Importantly *HoxC6* is also down regulated in XFD injected embryos. Thus the loss of anterior structures in embryos caused by the overexpression of *eFGF* after the MBT probably results from ectopic activation of the Hox genes in anterior regions. It has also been reported that FGFs can act as direct inducers of posterior neural tissue in gastrula ectoderm following brief disaggregation (Kengaku and Okamoto, 1995) or culture in low Ca^{++} and Mg^{++} medium (Lamb and Harland, 1995). Interpretation of these studies showing direct neuralisation by FGF is however, complicated by the fact that it has been shown that disaggregation of *Xenopus* ectoderm can directly lead to neural differentiation (Godsave and Slack, 1989; Grunz and Tacke, 1989). It is therefore possible that in these studies that there is some sub-threshold neuralisation caused by the culture conditions and that the basic phenomenon is still a posteriorisation of anterior neural tissue.

Interestingly the neural tissue induced by the noggin and follistatin proteins is of an anterior character (Lamb *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994). However, noggin-type neural inductions can be made more posterior in character by treatment with FGF during gastrula stages (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). Thus noggin may be likened to the activating principle and FGF to the transforming principle in Nieuwkoop's "activation-transformation" model of neural development (Nieuwkoop *et al.*, 1985). In recent years that has been much discussion as to the relative importance of "planar" versus

"appositional" signals involved in the induction and patterning of the nervous system. Further work will required to determine if the posteriorising influence of the FGFs *in vivo* is transmitted through the plane of the neural plate or through vertical signals from the underlying axial mesoderm. The expression pattern of *eFGF* is consistent with both possibilities.

The close relationship between the FGFs and *Brachyury* function raises the issue of what is the role of *Brachyury* in anteroposterior development? In the vertebrates *Brachyury* is expressed in 2 different domains in the posterior of the embryo and in the notochord. It has been suggested that the notochord domain corresponds to the *Brachyury* expression found in the common ancestor of the chordates (Reuter, 1995; Yasuo *et al.*, 1995). If so the expression of *Brachyury* in the posterior of vertebrate embryo would represent a novel domain. Analysis of the *no tail* mutant shows that trunk development is rather normal apart from the failure of the notochord to differentiate. However, there is a complete absence of tail development in *no tail* mutants. Therefore in terms of anteroposterior specification Brachyury is not required for the development of the trunk and head but is required for the development of the tail. On the other hand, FGF function is required for the development of the whole of the trunk and tail but not the head anterior of the midbrain. This strongly suggests that the requirement for FGF in formation of trunk somites and nervous system is independent of Brachyury. However, it seems likely that both FGF and Brachyury are required for the differentiation of the notochord. FGF is required for the formation of somites, nervous system and notochord during tail extension. However, in the tail this also requires the function of Brachyury. Data support the view that an important

function of Brachyury in the vertebrate tailbud is to maintain FGF expression and thus allow the production of the additional somites, notochord and posterior nervous system, which characterise the vertebrate tail. The existence of a positive feedback loop involving Brachyury and FGF complicates interpretation of the available data. Clearly both are required for normal tail development but is the only function of Brachyury to activate and maintain *eFGF* expression, probably by direct interaction with the *eFGF* promoter? It seems unlikely that *eFGF* is the only target of Xbra, however at present, there is no data showing that *Brachyury* can induce mesoderm or activate gene expression in the absence of a functional and perhaps downstream FGF signal transduction pathway. However, there is evidence that some of the changes in morphogenesis seen in zebrafish embryos that have been injected with *eFGF* mRNA take place in both wild-type and *no tail* embryos and are therefore independent of *Brachyury* function (Griffin *et al.*, 1995).

The FGFs and midline signalling

The presence of *eFGF* within the notochord also has implications for the signalling pathways involved in the development of other dorsal midline structures. Of particular interest is the fact that the signalling molecule *sonic hedgehog* (*shh*) has also been shown to be expressed in the notochord of *Xenopus*. *Shh* homologues have been identified in a number of vertebrate species and it has been implicated in several patterning processes in early development including the formation of feather buds, patterning of the limb and the induction of the floor plate and motor neurons in the ventral neural tube (Nohno *et al.*, 1995; reviewed by Pownall, 1994; Smith, 1994).

There is a considerable body of evidence which suggest that during limb development the activity of members of the FGF family in the apical ectodermal ridge (AER) is required to maintain the expression of *Shh* in the posterior mesenchyme of the limb bud. The candidate FGFs for this activity include *FGF-4* and *FGF-8* both of which are expressed in the AER and are able to maintain *shh* expression in the absence of the AER (Mahmood *et al.*, 1995a; Niswander *et al.*, 1994; Vogel *et al.*, 1995). Given the fact that *eFGF* is closely related to *FGF-4* it is interesting to speculate that *eFGF* and *shh* may interact in a similar way during the induction of dorsal midline structures. The two situations are not directly comparable because in the dorsal mid line, unlike the limb, *eFGF* and *shh* are initially expressed in the same tissue. Although *FGF-3* is not expressed in the notochord, it is expressed at low levels in the dorsal midline of the neural plate in cells which will contribute to the floor plate. It is therefore possible that *FGF-3* is also involved in the induction and patterning of dorsal midline structures.

A model for the role of the FGFs in amphibian development

It is now possible to outline a model for the role of FGFs during amphibian development. Much of the discussion in this thesis has concerned the activities of eFGF in development but it is quite likely that *in vivo* more than one FGF contributes to the processes described below. However, eFGF does provide a useful paradigm for secreted FGFs (such as *FGF-3*), which are expressed in overlapping domains of expression during early development. The scheme below relies heavily on data that highlight those processes in the embryo for which the activity of the FGF signalling pathway is necessary; clearly the involvement of

other factors in these processes cannot be excluded and indeed is to be expected. In particular, it has recently been shown that two novel ligands, unrelated to other members of the FGF family, can activate signal transduction by FGF-R1 and are expressed during early *Xenopus* development (Kinoshita *et al.*, 1996). Furthermore, demonstration that cell adhesion molecules can specifically interact with FGF receptors suggests that direct cell-cell contacts are likely to be important in regulating the activity of the FGF pathway (Williams *et al.*, 1994). The activities of the FGFs can also be modified by other growth factor-like molecules, which are known to be present in the developing embryo. So it is to be expected that the FGF signal transduction pathway interacts in a complex fashion with that of members of the TGFß, Wnt, chordin and noggin families of secreted factors (reviewed by Kimelman *et al.*, 1992; Slack, 1994; Holley *et al.*, 1995).

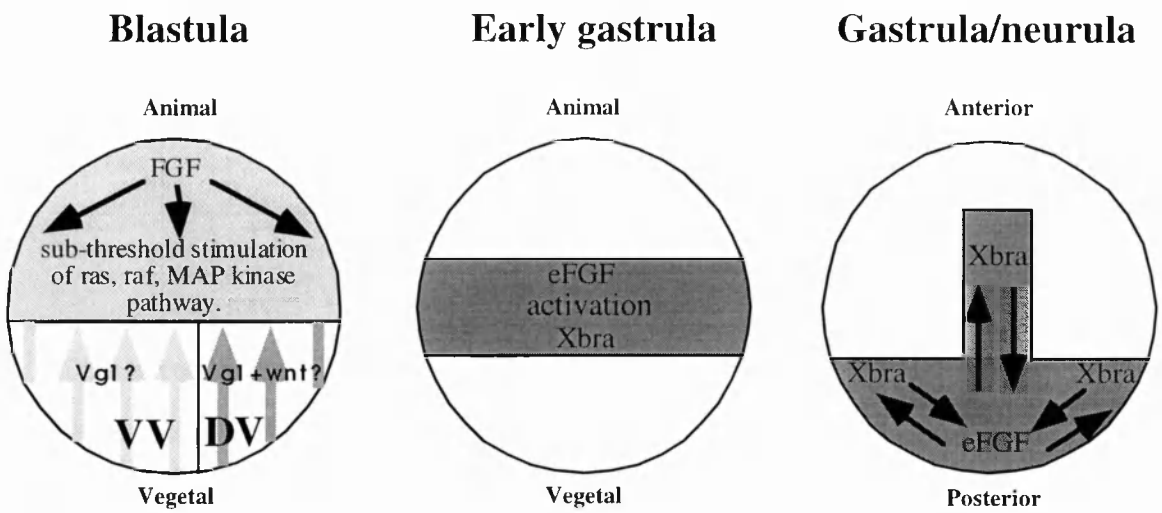
Early models of FGF function during development suggested that they may act as vegetally localised mesoderm inducing molecules. This now seems unlikely because the known FGFs in *Xenopus* are more abundant in the animal hemisphere. However, experiments with the dominant negative FGF receptor indicate that FGF activity is required during blastula stages for certain aspects of mesoderm formation. Current data support the notion that the maternal pool of FGFs is required to provide sub-threshold stimulation of the tyrosine kinase signal transduction pathway in the animal hemisphere. The maternal FGFs may be viewed as competence factors which need to be present for the full range of responses to the vegetally localised inducing molecules. It is likely that a major component of the vegetal signal is an activin-like molecule such as vg1 (Dale *et al.*, 1993; Thomsen and Melton, 1993), perhaps acting in combination with a

member of the Wnt family (Heasman *et al.*, 1994; He *et al.*, 1995). Cornell and Kimelman have proposed that the mesoderm forms in the marginal zone because it is only here that cells are exposed to both an FGF and an activin-like signal (Cornell *et al.*, 1995). Absence of FGF "competence" activity from the vegetal hemisphere explains why the whole of the vegetal hemisphere does not mesodermalise in response to the endogenous inducing signals and at the same time explains why inhibition of the activin signalling pathway blocks the formation of all mesoderm.

Of particular importance is that a low level of maternal FGF activity is required for the transcription of *Xbra* in the marginal zone of the late blastula. The zygotic expression of *eFGF* is also activated in the marginal zone. At present it is not known if the expression of *eFGF* is an immediate early response to the vegetal inducer, as is the case for *Xbra*, or whether the zygotic expression is only activated by Xbra. During this phase eFGF may function as a secondary mesoderm inducing factor which amplifies and spreads the effect of the primary vegetal inducers, resulting in activation and maintenance of gene expression, including *Xbra*, within the marginal zone, and in this way contributing to the stability of the mesoderm. In the gastrula a positive feedback loop operates between eFGF and Xbra, which helps maintain their normal levels of expression. The expression of both of these genes is activated in response to activin and the proposed positive feedback loop would provide one mechanism for the generation of the sharp thresholds of *Xbra* activation seen in animal cap cells treated with activin (Green *et al.*, 1992). In gastrula and neurula stages the feedback loop continues to regulate the expression of *Xbra* and *eFGF* in the blastopore region

and possibly also in the notochord. A summary of the proposed model for the role of the FGFs in regulating the expression of *Xbra* is shown in Fig. 1.

Fig.1 FGFs and the regulation of *Xbra* expression during early *Xenopus* development



The activity of the FGFs is also required for the correct regulation of other genes expressed in the mesoderm, most likely on parallel pathways to that of *Xbra*. FGF function is necessary for the correct development of *XmyoD* expression, which indicates a role in the development of the myogenic lineages. In addition to activities within the early mesoderm there is evidence that the FGFs have a number of activities in the ectoderm. *Xsna* expression in the gastrula ectoderm is an early marker of neural crest. In XFD injected embryos the ectodermal expression of *Xsna* is absent indicating that FGF activity is required for crest formation.

Furthermore there is now good evidence that the FGFs function as posteriorising agents involved in the anteroposterior specification of the neuroectoderm acting through the activation of a subset of posterior Hox genes.

During tailbud extension FGF activity continues to be required for the formation of the posterior nervous system, notochord and somites.

Origins of the dominant negative phenotype

In embryos injected with the dominant negative FGF receptor there are characteristic effects on tissue specification, gene expression and cell movements. Can the phenotype of XFD injected Xenopus embryos be accounted for solely by an interference with the gene regulatory pathways outlined above? Or does the loss of trunk and tail structures mainly result from aberrant dorsal cell movements? At present this is a very hard question to answer given the intimate link between specification, patterning and morphogenesis in vertebrate development. However, the involvement of the FGFs in the gene regulatory pathways described above is well supported both by examination of gene expression in embryos lacking a functional FGF signal transduction pathway and biochemical data from a number of *in vitro* assays. It is likely that an interference with these pathways can to a large extent account for the alteration in tissue specification and phenotype seen in XFD injected embryos.

Lessons from the mouse knockouts

Close homologues of the molecules discussed in this account are present in other organisms and, given the high conservation of developmental mechanisms between vertebrate species, it is likely that much of what has been learnt concerning the targets and regulation of FGF activity in *Xenopus* will be generally applicable. In mouse targeted gene disruption has been carried out for *KGF*

(*FGF-7*), *FGF-3* (*int-2*), *FGF-4* and *FGF-5*. The latter 3 ligands are expressed in the primitive streak and therefore likely to be involved in the processes outlined for the FGFs in *Xenopus* .

FGF-5 knockout mice are viable have abnormally long coat hair and it is believed that the mouse mutant *angora* results from a naturally occurring mutation in the *FGF-5* gene (Hebert *et al.*, 1994). *FGF-7* (*KGF*) null mutant mice are also viable and also show abnormalities in hair growth. Over time their coats become very matted and are similar in appearance to the *rough* mutant mouse. Perhaps unexpectantly given the potent activity of KGF as a mitogen for keratinocytes these mice show no abnormalities in epidermal growth or wound healing (Guo *et al.*, 1996). Mice embryos homozygous for a null mutation in the *FGF-3* gene are viable but do not survive until adulthood. Interestingly in context of the likely role of FGF in posterior development homozygous *FGF-3* null mice have abnormally short and curved tails. FGF-4 is the murine FGF most closely related to eFGF. However, homozygous FGF-4 null mutant mice are early embryonic lethals and do not even reach primitive streak formation. It is therefore not possible to tell if FGF-4 has similar function to that proposed for eFGF during amphibian gastrulation. Lethality appears to be due to a reduction in cell proliferation in the cells of the inner cell mass (ICM). This emphasises an important difference between the early development of the amphibia and the amniotes. During the early development of the amphibia there is no growth in size but early tissue specification and patterning in the higher vertebrates is accompanied by substantial growth. The data from the FGF-4 knockout strongly suggest that there is a requirement for FGF activity to stimulate growth of the inner cell mass before the onset of

gastrulation and the homologous patterning events described in this thesis (Feldman *et al.*, 1995). Such early growth requirements will necessarily complicate the analysis of FGF function in the amniotes and makes it likely that the amphibian model will continue to provide useful insights into the role of the FGFs in vertebrate development.

The phenotype of FGF-R1 null mutants is somewhat different to that seen with the XFD injections in *Xenopus*, because, in addition to frequent posterior truncations, development of the head structures is also often reduced. Again the interpretation of the phenotype is complicated by the requirement for FGFs in cell proliferation during mouse embryogenesis. However, significantly, the expression of *Brachyury* is not reduced in *FGF-R1* null mutants and if anything there is an expansion of the *Brachyury* notochord domain of expression. Clearly the potential for redundancy of gene function in the multigene FGF ligand and receptor families complicates matters greatly. Thus it is likely that *FGF-R2*, which is also expressed during gastrulation, is able to mediate FGF regulation of *Brachyury* in FGF-R1 null mutants. The effects produced by the overexpression of the dominant negative FGF receptor in *Xenopus* are very dramatic because it is likely that this approach inhibits the function of many if not all members of the FGF family.

The phenotype of mice homozygous for a null mutation in the *FGF-R3* has also been recently reported (Deng *et al.*, 1996). *FGF-R3* is expressed at high levels in the cartilage during the development of a wide range of bones in the mouse (Peters *et al.*, 1993). *FGF-R3* mutants are viable but consistent with this expression pattern null mutants show a overgrowth of skeletal structures, including the vertebrae and the long bones. This apparently arises from an over

proliferation of chondrocytes suggesting that signalling from FGF-R3 acts as a negative regulator of bone growth. Interestingly a number of mutations have been identified in the human *FGF-R3* gene which are associated with dominantly acting skeletal dysplasias in humans, achondroplasia and hypochondroplasias (reviewed by Muenke and Scell, 1995). The dwarf phenotype of patients carrying these mutations, which can be in several different regions of the receptor, including the extracellular domain and kinase domains, is the opposite of bone overgrowth seen in the FGF-R3 null mice. This suggests that these mutations are probably gain of function and involve some form of ligand independent activation of *FGF-R3*. Mutations in *FGF-R1* and *FGF-R2* have also been shown to be associated with other human dominant skeletal dysplasias, including Pfeiffer, Crouzon and Apert's syndromes (Muenke *et al.*, 1994; Reardon *et al.*, 1994; Wilkie *et al.*, 1995). These clearly indicate a role for the FGF family in skeletal development but give no indication of the role of FGF signalling during early development.

The knockout of the *FGF-4* gene is an early embryonic lethal and this suggests that generalised transgenic overexpression of a dominant negative FGF receptor during early mouse development is also likely to be an early embryonic lethal. In order to overcome this problem a number of transgenic studies have been carried out using a dominant negative receptor driven off a strong region specific promoter. Using this approach it has been shown that FGF function is required for branching morphogenesis in the developing lung (Peters *et al.*, 1994) and for the normal pattern of keratinocyte differentiation in the skin (Werner *et al.*, 1993).

At present the results obtained from single gene knockouts have not been particularly illuminating as to the conservation of the roles of the FGFs between *Xenopus* and the higher vertebrates, during the very early patterning processes of development. It is to be hoped that this situation will improve with the generation of multiple ligand and receptor null mutants and conditional knockouts, which will allow improved temporal and regional control over the disruption of gene function during development.

However, despite the insights that will be gained from the study of transgenic mice, the many of advantages of the amphibian embryo, for the study of early events in regional specification, will guarantee that work in *Xenopus* will continue to contribute to our understanding of the requirement for FGF signalling during development.

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